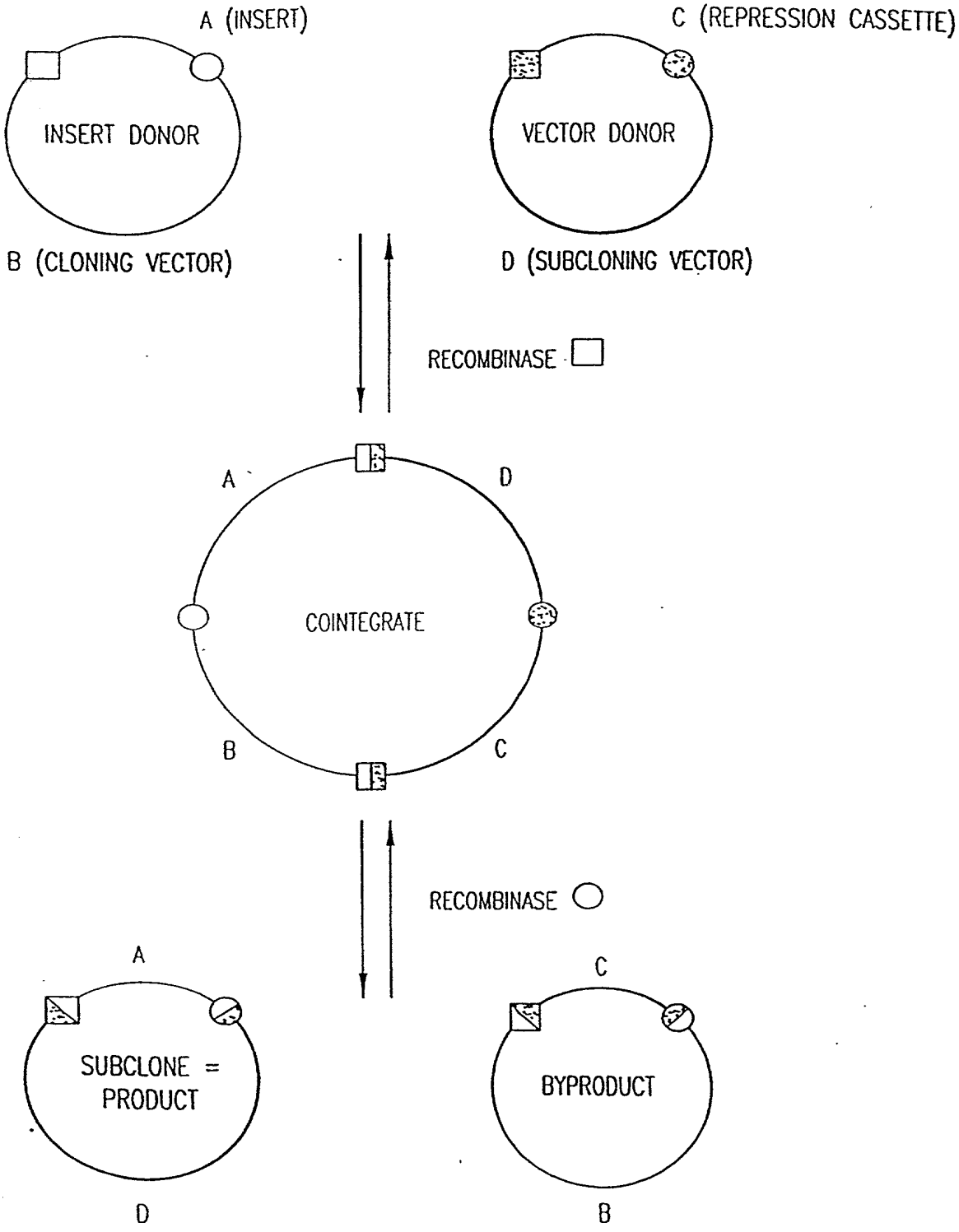

A (INSERT)
C (REPRESSION CASSETTE)
INSERT DONOR
VECTOR DONOR
B (CLONING VECTOR)
D (SUBCLONING VECTOR)
RECOMBINASE
A
D
COINTEGRATE
B
C
RECOMBINASE
A
C
SUBCLONE = PRODUCT
BYPRODUCT
D
B

FIG. 1

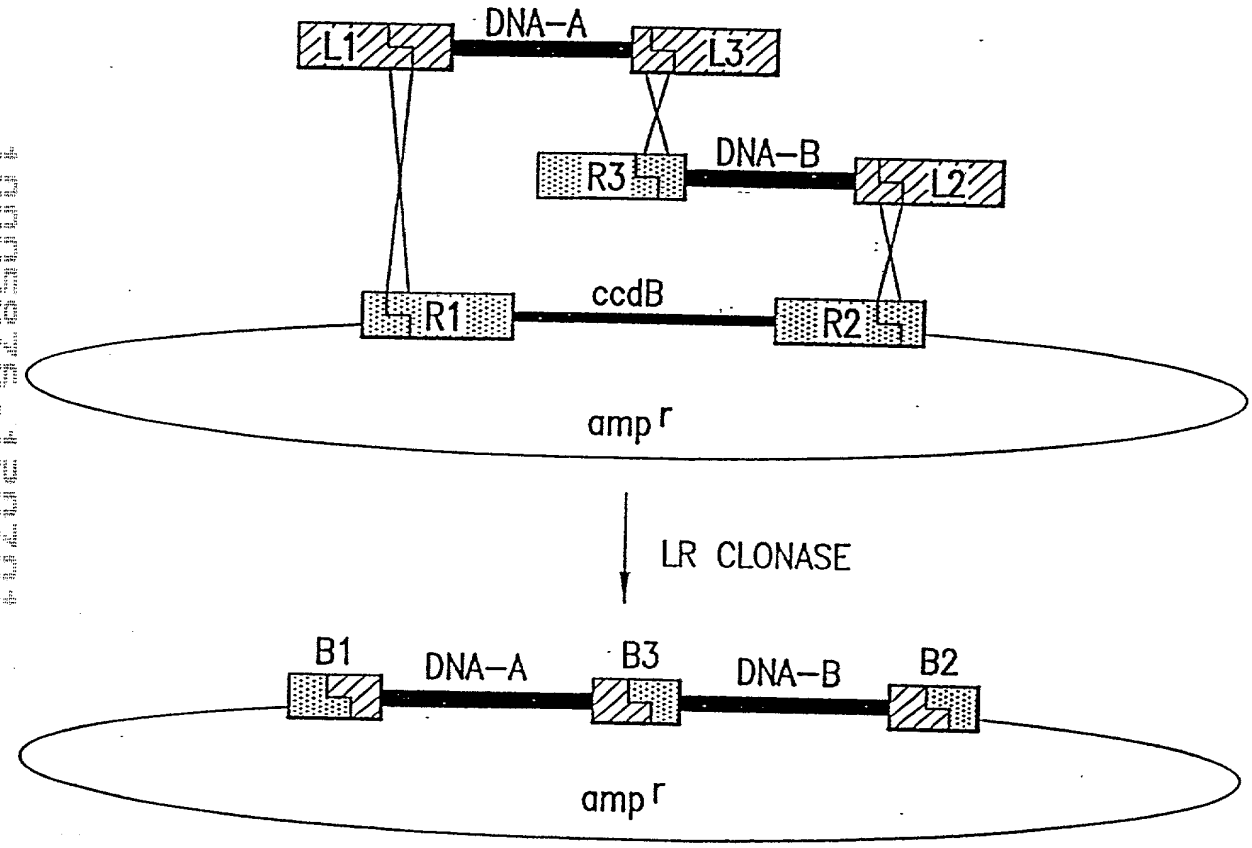


FIG.2

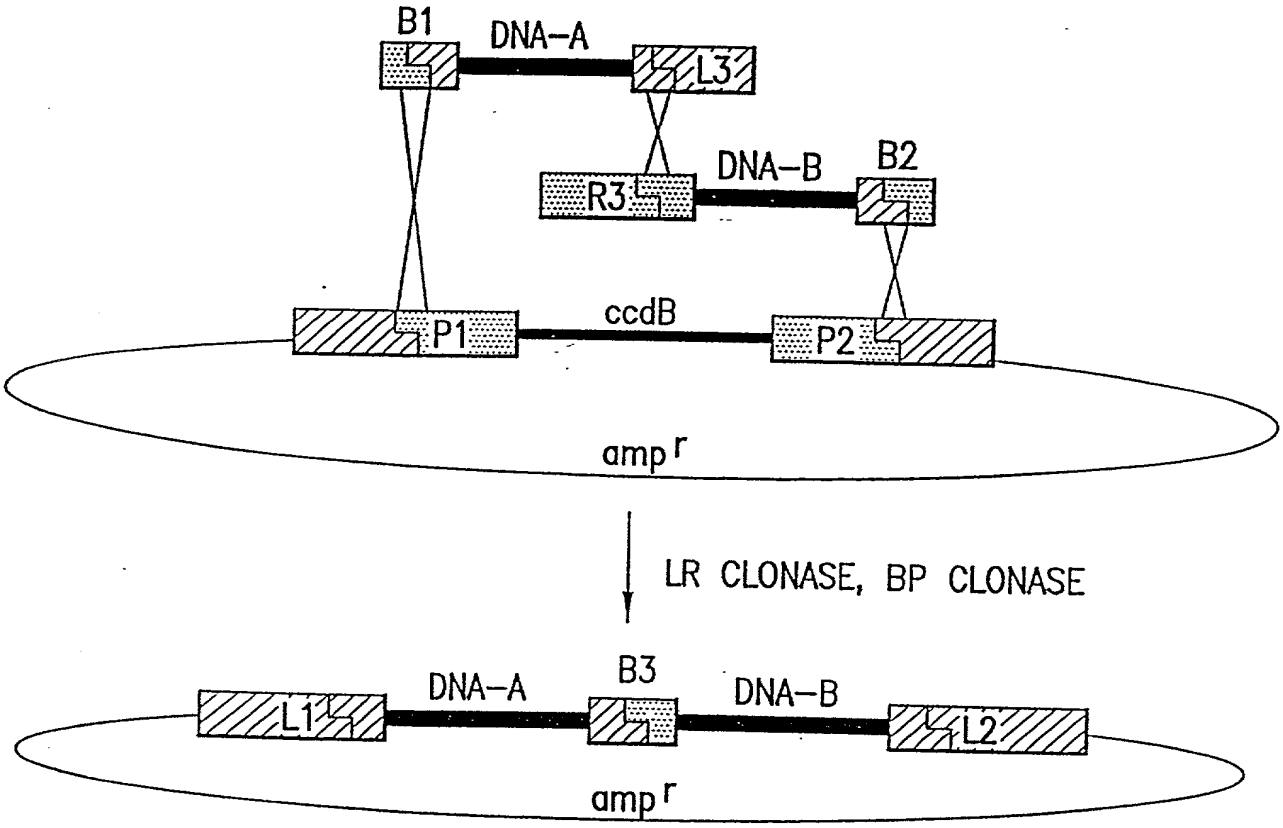


FIG.3

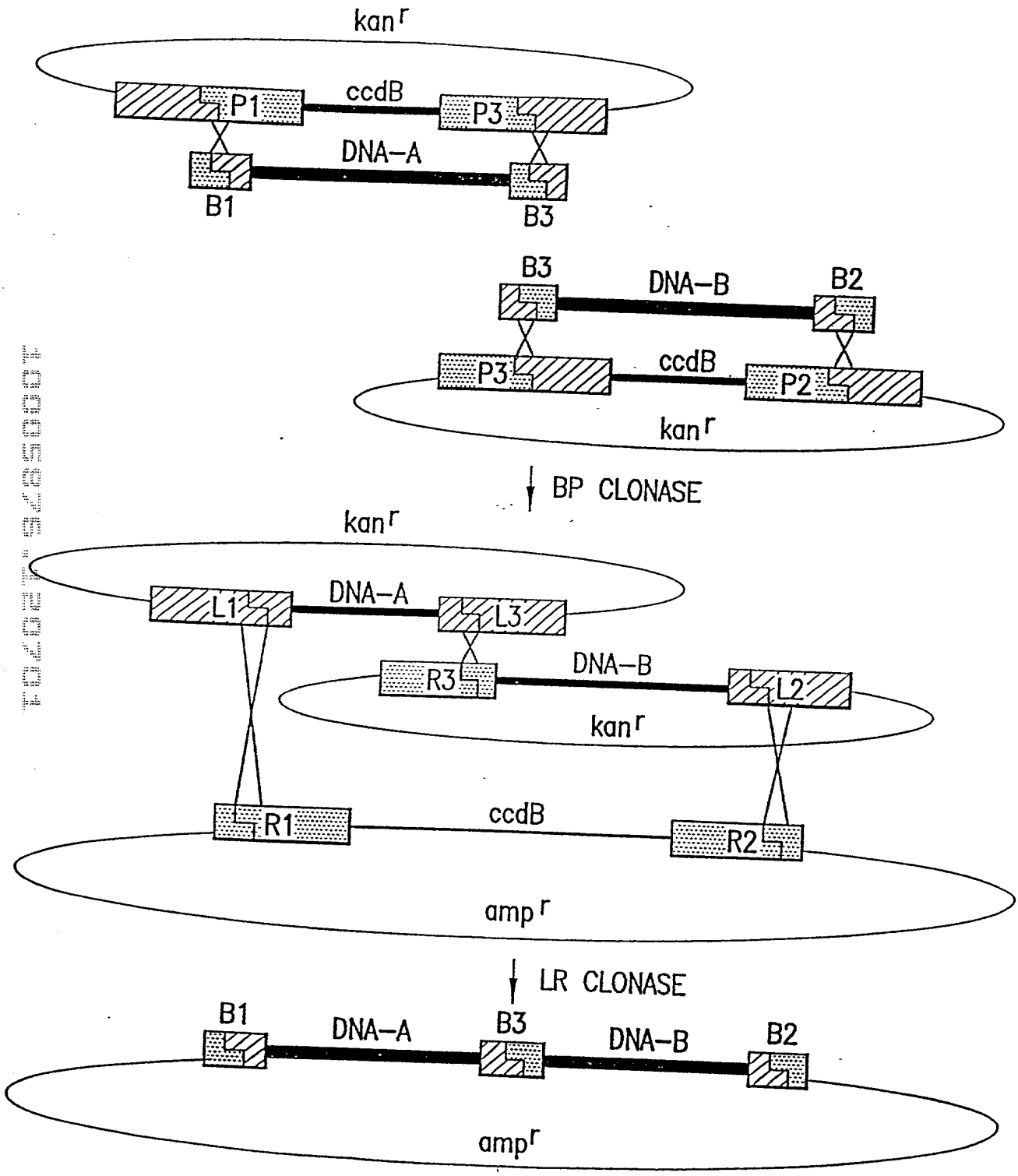

kan r
P1
ccdB
P3
DNA–A
B1
B3
B3
DNA–B
B2
P3
ccdB
P2
kan r
BP CLONASE
kan r
L1
DNA–A
L3
R3
DNA–B
L2
kan r
R1
ccdB
R2
amp r
LR CLONASE
B1
DNA–A
B3
DNA–B
B2
amp r

FIG.4

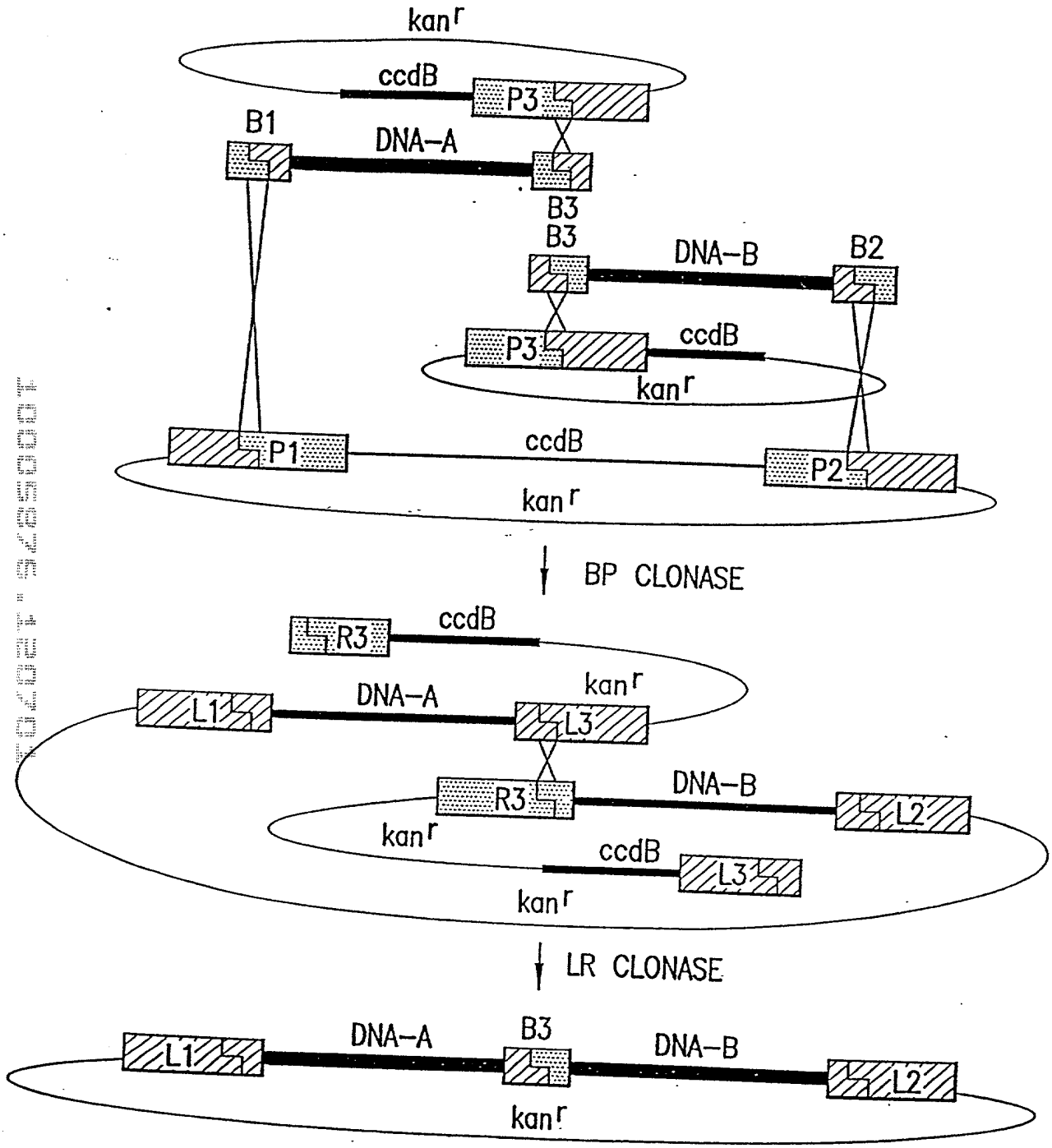

kan r
ccdB
P3
B1
DNA–A
B3
B3
DNA–B
B2
P3
ccdB
kan r
P1
ccdB
P2
kan r
BP CLONASE
R3
ccdB
kan r
L1
DNA–A
L3
R3
DNA–B
L2
kan r
ccdB
L3
kan r
LR CLONASE
L1
DNA–A
B3
DNA–B
L2
kan r

FIG.5

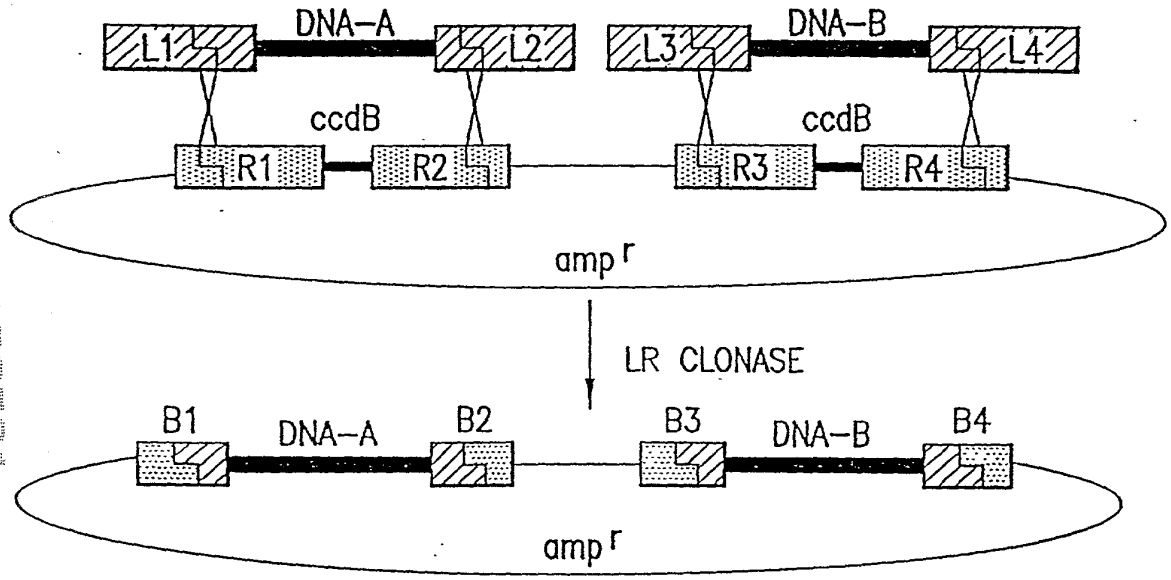


FIG.6

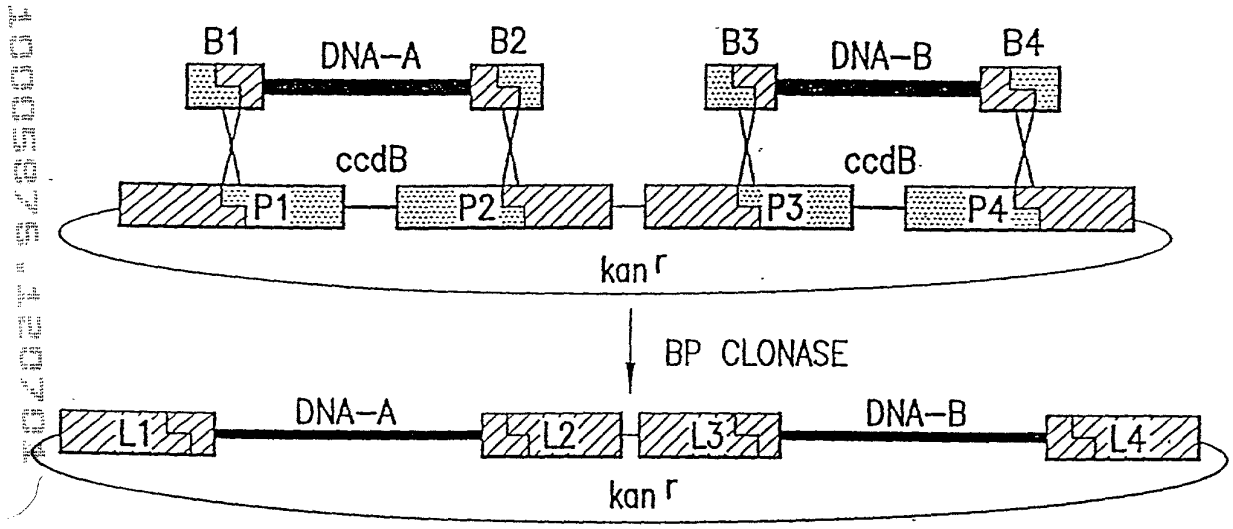

B1
DNA–A
B2
B3
DNA–B
B4
ccdB
ccdB
P1
P2
P3
P4
kan r
BP CLONASE
L1
DNA–A
L2
L3
DNA–B
L4
kan r

FIG.7

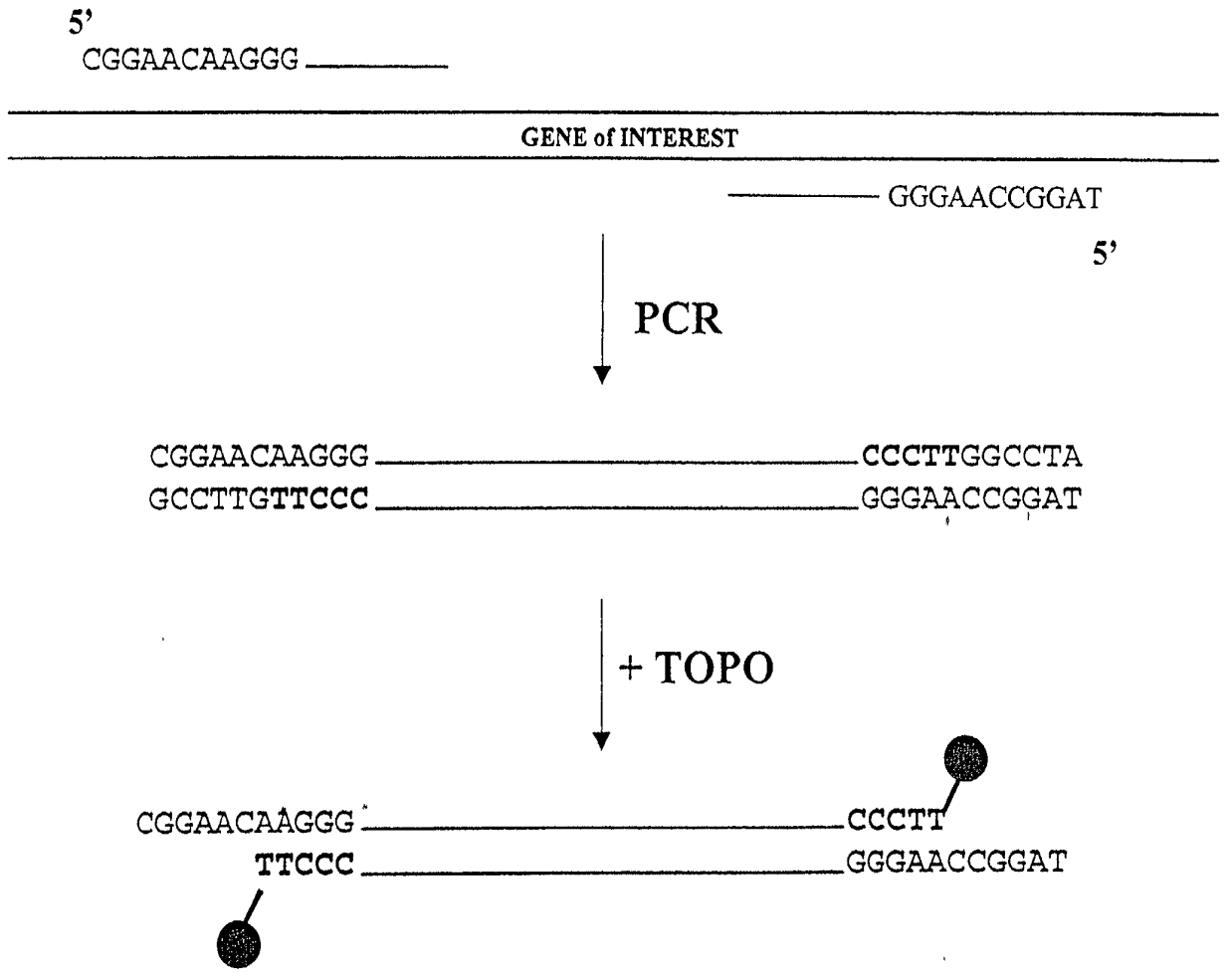

5'
CGGAACAAGGG
GENE of INTEREST
GGGAACCGGAT
5'
PCR
CGGAACAAGGG
CCCTTGGCCTA
GCCTTGTTCCC
GGGAACCGGAT
+ TOPO
CGGAACAAGGG
CCCTT
TTCCC
GGGAACCGGAT

FIGURE 8A

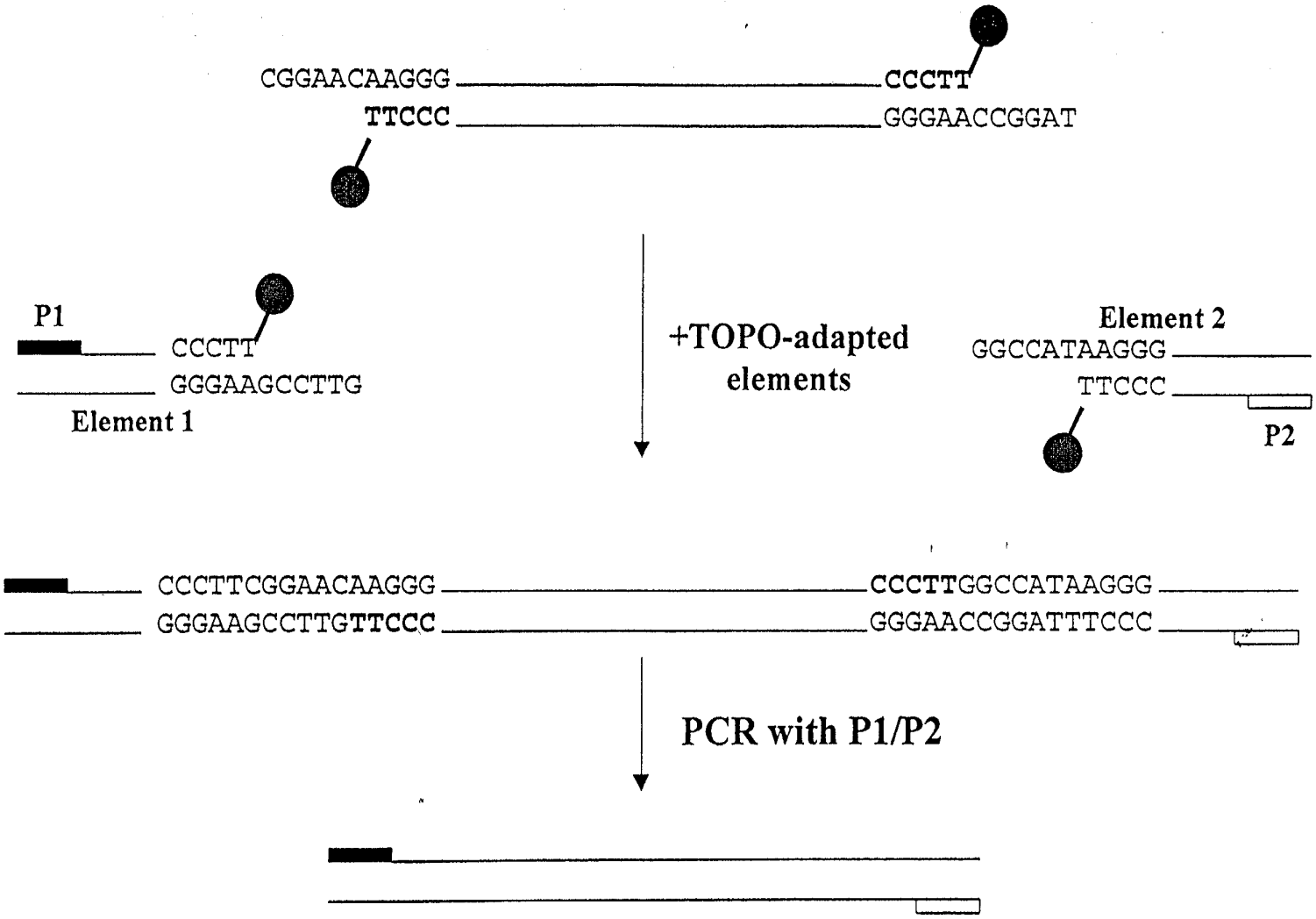

CGGAACAAGGG
CCCTT
TTCCC
GGGAACCGGAT
P1
CCCTT
GGGAAGCCTTG
Element 1
+TOPO-adapted elements
Element 2
GGCCATAAGGG
TTCCC
P2
CCCTTCGGAACAAGGG
CCCTTGGCCATAAGGG
GGGAAGCCTTGTTCCC
GGGAACCGGATTTCCC
PCR with P1/P2

FIGURE 8B

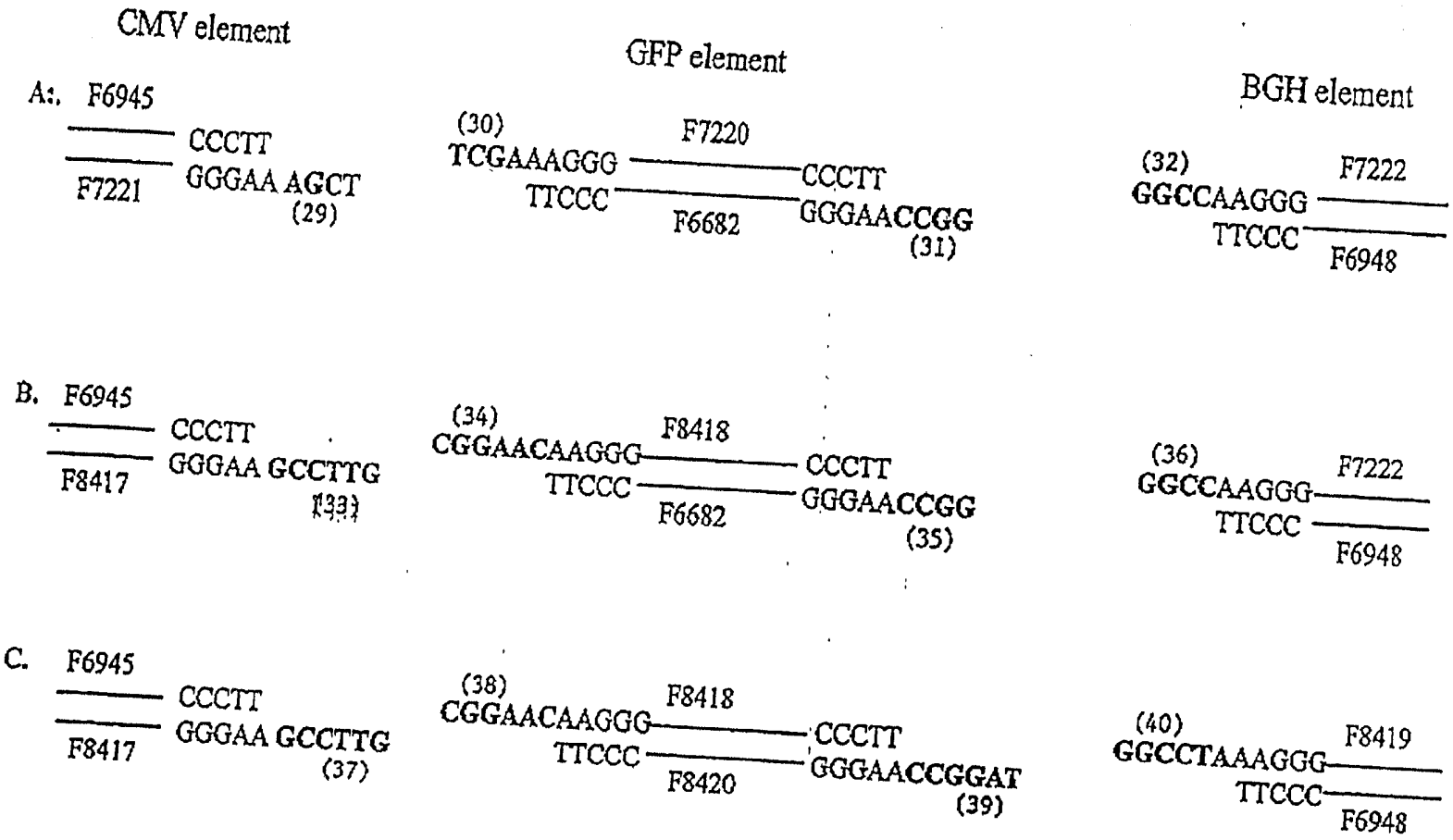


FIGURE 9A-C

Table 1

Primer name	F#	Sequence (5' -> 3')	SEQ ID NO:
MTH1	10779	TATGTATCATACACATACGATTTAGGT	1
MTH2	10780	ACCGCCTCTCCCCGCGCGTT	2
GAL4r2	12667	GTTCCGAAGGGGGCGATACAGTCAACTGTCTTTG	3
MTH5	12505	TTGGCCAAGGGTATCTAGAAGCTTCTGCAGACGCGT	4
VP16r2	12668	GTTCCGAAGGGCCACCGTACTCGTCAATTCCAAG	5
SV40pAf	12016	GGCCAAAAGGGAACTTGTTTATTGCAGCTTATAATG	6
SV40pAr	561	CTCTGACTTGAGCGTCGATTTT	7
p53f2	12669	CGGAACAAGGGGAATTCCCTGTCACCGAGACC	8
SVTf2	12670	CGGAACAAGGGGAATTCCCGGGGATCTGGAATTC	9
CMVr2	7221	TCGAAAGGGTCGAGGTCGACCTGCAGCTG	10
CMVf	6945	AATTCACATTGATTATTGAGTAGTTA	11
GFP-Xhof	7220	TCGAAAGGGTAATGGCCAGCAAAGGAGAAG	12
GFP-Notr	6682	GGCCAAGGGTTTGTAGAGCTCATCCAT	13
BGHf2	7222	GGCCAAGGGTCTGAATGGGGCCGCATAGT	14
BGHr	6948	AAGCCATAGAGCCCGGGCCA	15
CMVr3	8417	GTTCCGAAGGGTCGAGGTCGACCTGCAGCTG	16
GFPf3	8418	CGGAACAAGGGATGGCCAGCAAAGGAGAAG	17
GFPr3	8420	TAGGCCAAGGGTTTGTAGAGCTCATCCATGC	18
BGHf3	8419	GGCCTAAAGGGTGAATGGGGCCGCATAGT	19
T7top	9304	GAAGGAGTAATACGACTCACTATAGGGAGCCACCATGGGCCCTTCGGAAC	20
T7bottom	9305	GTTCCGAAGGGCCCATGGTGGCTCCCTATAGTGAGTCGTATTACTCCTTC	21
T7amp	9306	GAAGGAGTAATACGACTCACT	22
T3top	9661	GGCCTAAAGGGTCCCTTTAGTGAGGGTTAATTGCGCGC	23
T3bottom	9662	GCGCGCAATTAACCCTCACTAAAGGGACCCTTTAGGCC	24
lacZf2	10632	CGGAACAAGGGATGATAGATCCCGTCGTTTTACA	25
lacZ1k2	10770	TAGGCCAAGGGGACCATTTTCAATCCGCACCT	26
lacZ2k2	10771	TAGGCCAAGGGGAGGCACTTCACCGCTTGCCA	27
lacZ3k2	10772	TAGGCCAAGGGTTTGACACCAGACCAACTGGTA	28

FIGURE 9D

A.

Sample #	GAL4+pA	VP16+pA	pGene/lacZ	GAL4+p53+pA	VP16+T+pA	p53-VP16
1			0.26 ug	p 0.37 ug	p 0.37 ug	
2			0.4 ug	p 0.3 ug	p 0.3 ug	
3			0.4 ug			p 0.6 ug
4			0.4 ug	T 0.3 ug	T 0.3 ug	
5		T 0.3 ug	0.4 ug	T 0.3 ug		
6	T 0.3 ug		0.4 ug		T 0.3 ug	
7			0.4 ug	4.5 ul PCR	4.5 ul PCR	
8		4.5 ul PCR	0.4 ug	4.5 ul PCR		
9	4.5 ul PCR		0.4 ug		4.5 ul PCR	

B.

Sample #	LacZ activi
1	240000
2	140000
3	1800000
4	1400000
5	54000
6	80000
7	320000
8	12000
9	42000

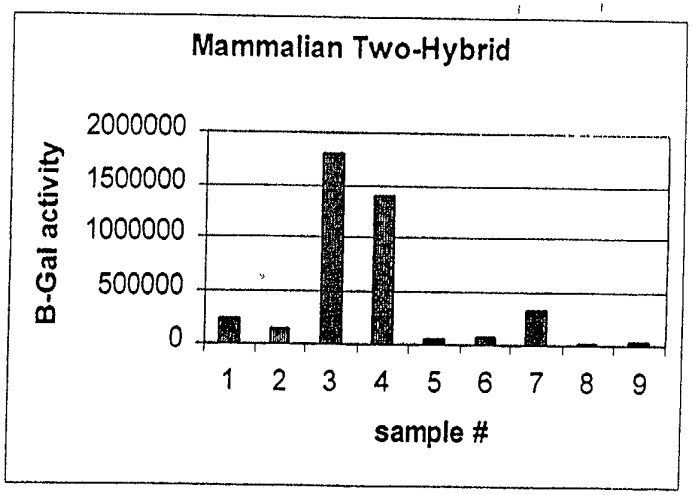


FIGURE 10

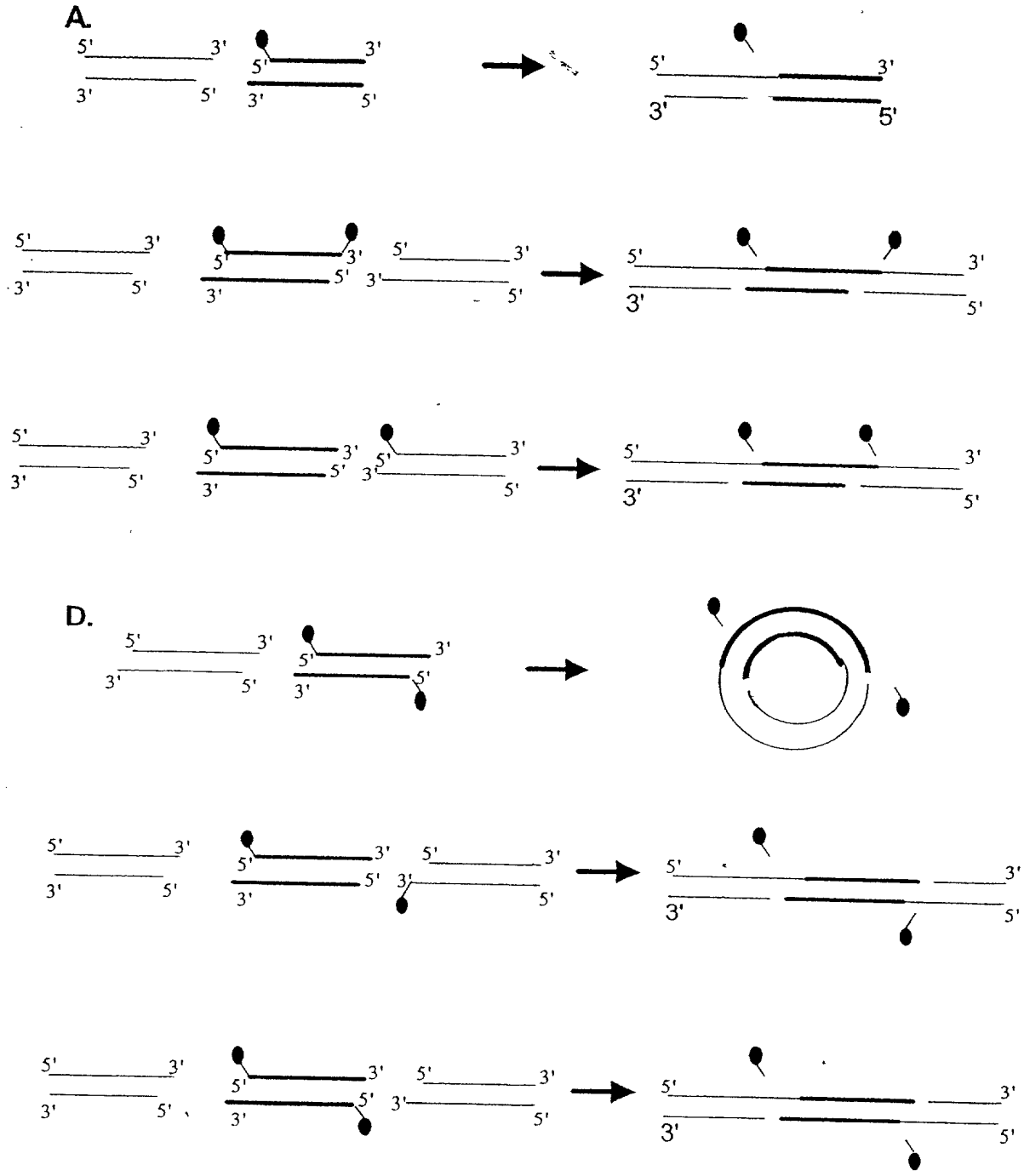


FIGURE 11

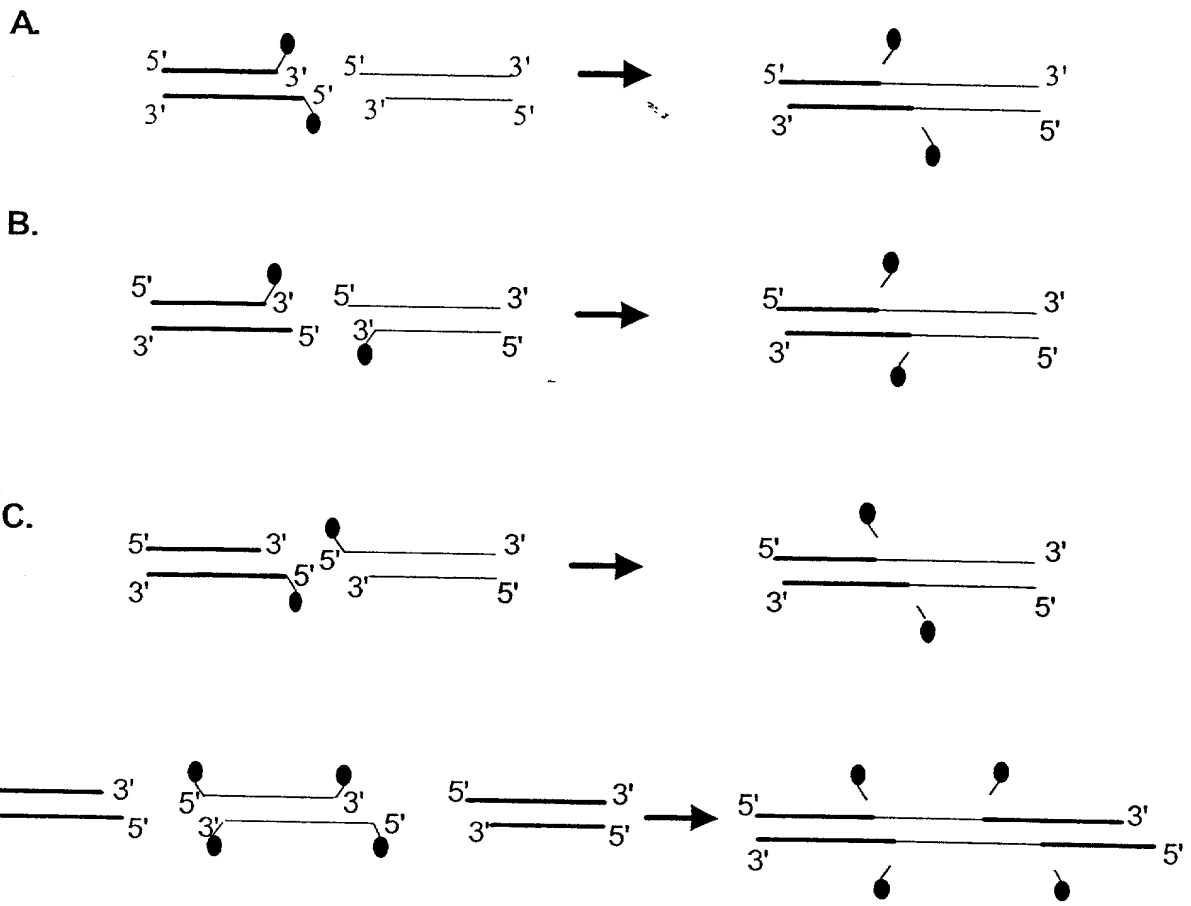

A.
5'
3'
5'
3'
3'
5'
5'
3'
5'
3'
B.
5'
3'
5'
3'
3'
5'
3'
5'
5'
3'
3'
5'
C.
5'
3'
5'
3'
3'
5'
5'
3'
3'
5'
5'
3'
3'
5'
D.
5'
3'
3'
5'
5'
3'
3'
5'
5'
3'
3'
5'
5'
3'
3'
5'

FIGURE 12

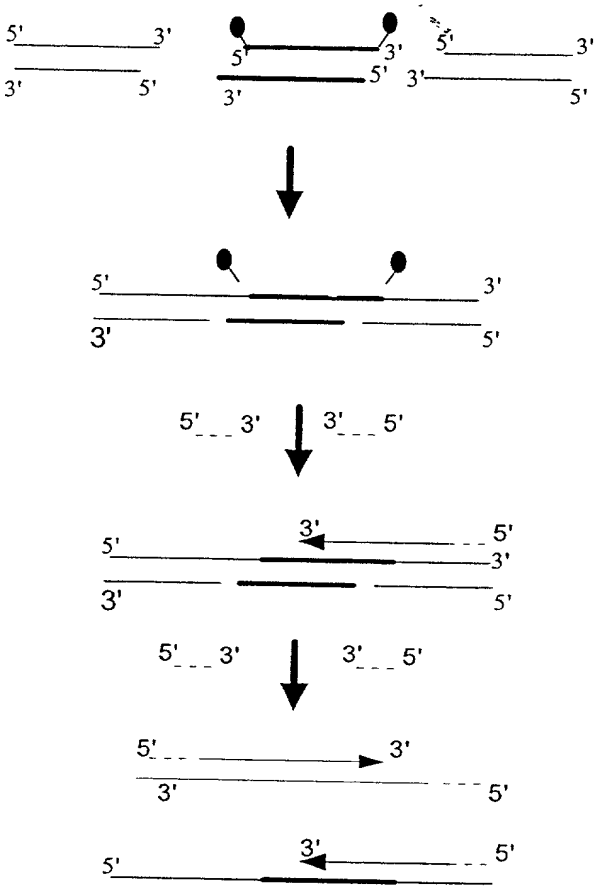


FIGURE 13

FIGURE 14

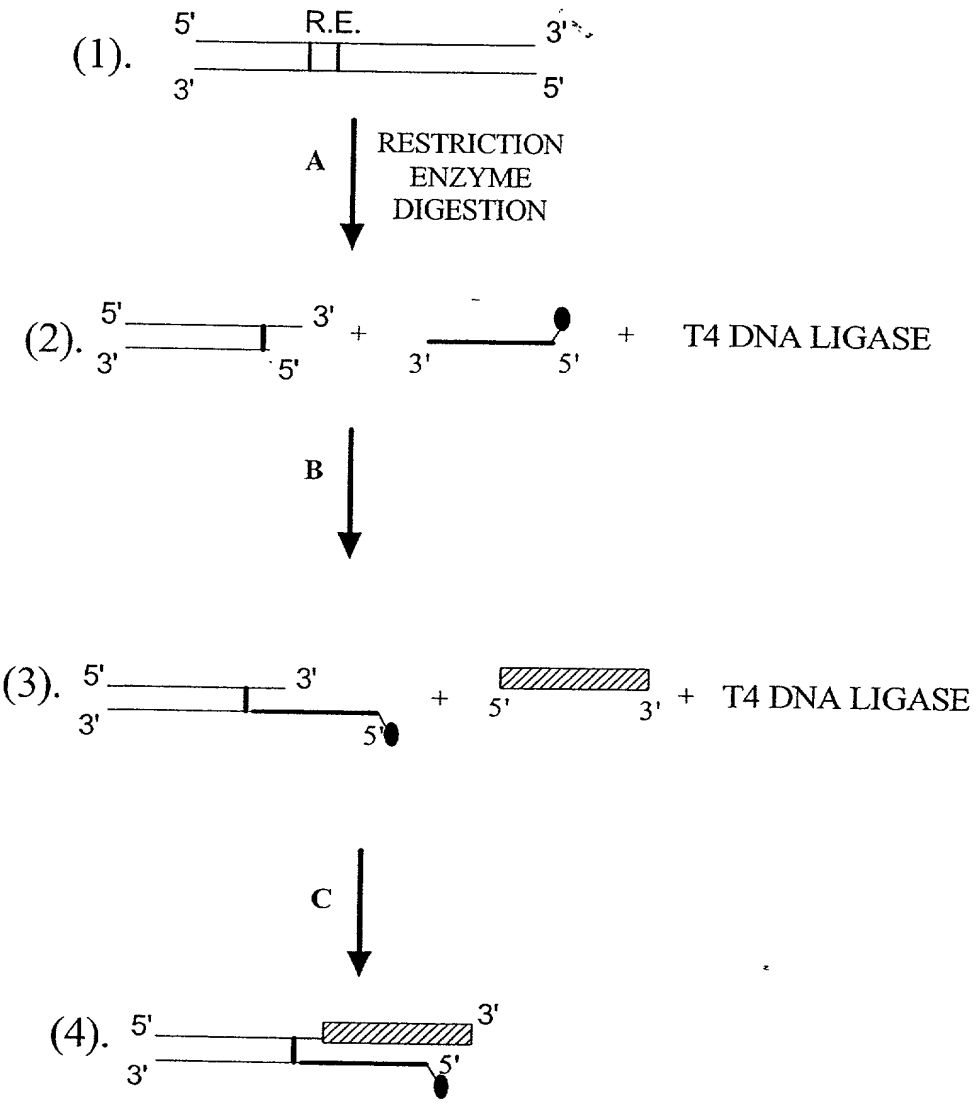
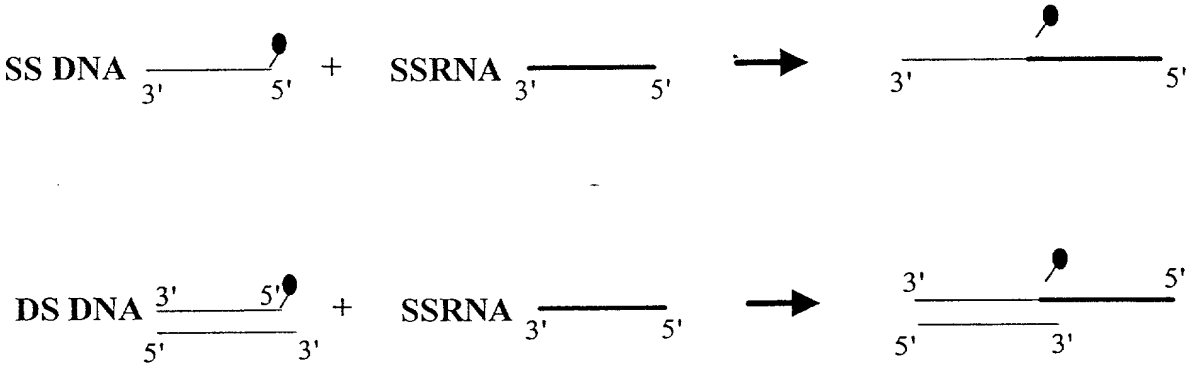

(1).
5'
R.E.
3'
3'
5'
A
RESTRICTION
ENZYME
DIGESTION
(2).
5'
3'
3'
5'
+
3'
5'
+
T4 DNA LIGASE
B
(3).
5'
3'
3'
5'
+
5'
3'
+
T4 DNA LIGASE
C
(4).
5'
3'
3'
5'

FIGURE 15



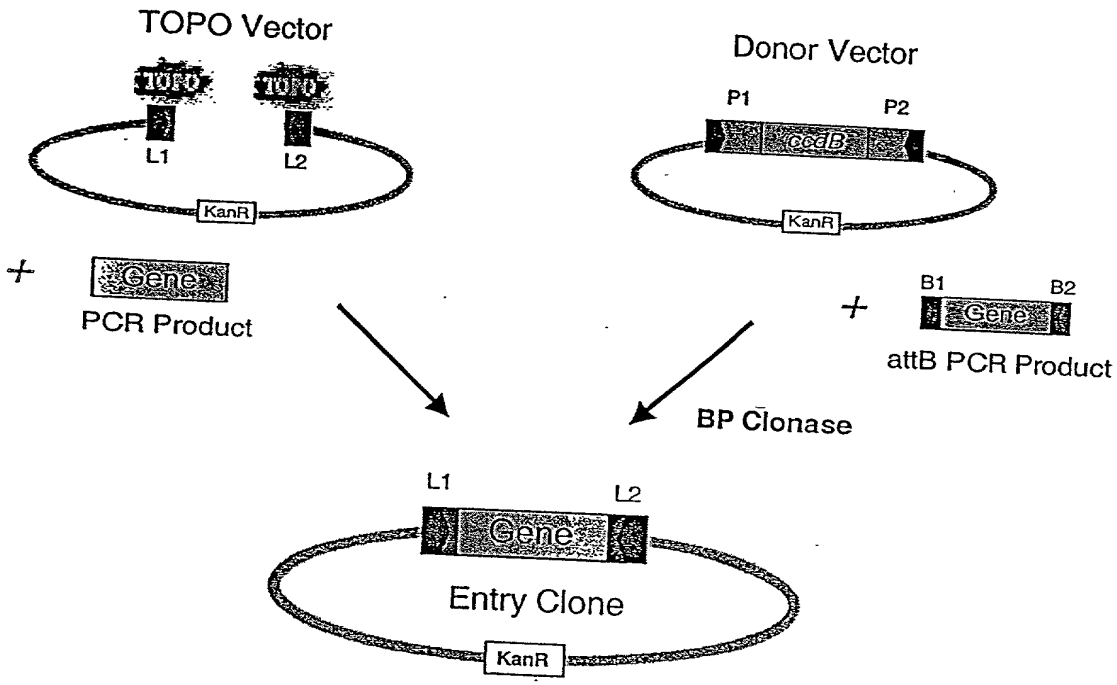

TOPO Vector
TOPO
TOPO
L1
L2
KanR
+
Gene
PCR Product
Donor Vector
P1
P2
ccdB
KanR
+
B1
B2
Gene
attB PCR Product
BP Clonase
L1
L2
Gene
Entry Clone
KanR

FIGURE 16

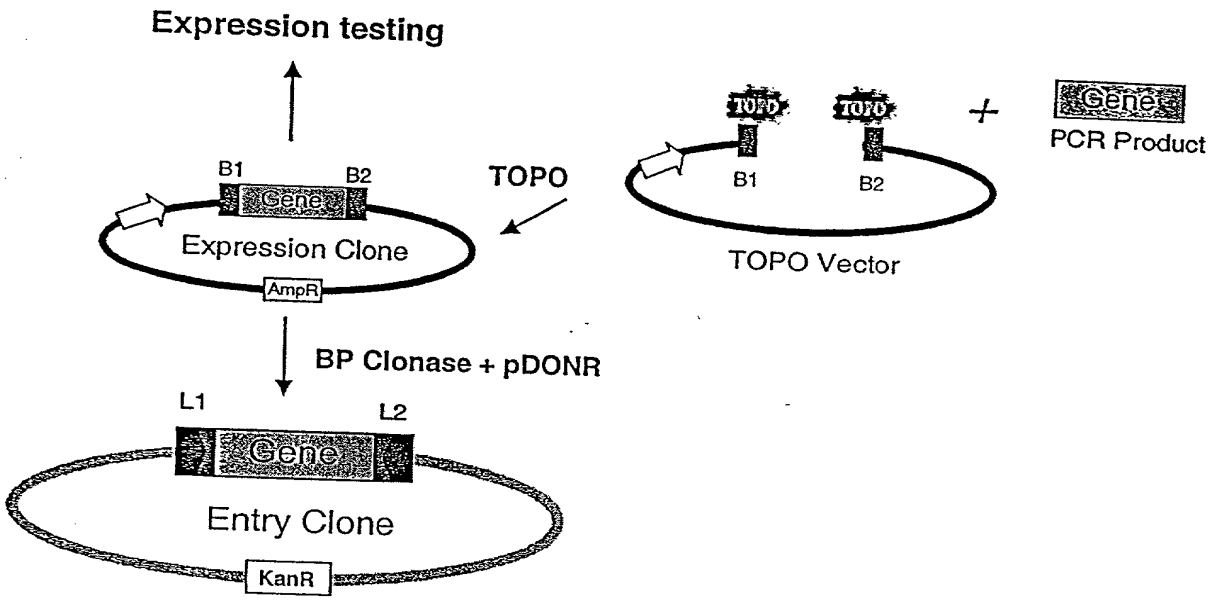

Expression testing
B1
B2
Gene
Expression Clone
AmpR
TOPO
TOPO
TOPO
B1
B2
TOPO Vector
+
Gene
PCR Product
BP Clonase + pDONR
L1
L2
Gene
Entry Clone
KanR

FIGURE 17

MCS for pcDNAGW-DT(sc) and pENTR-DT(sc)

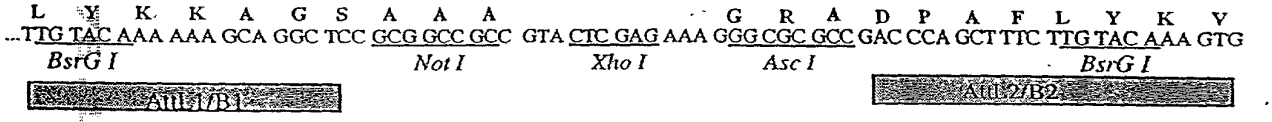


FIGURE 18

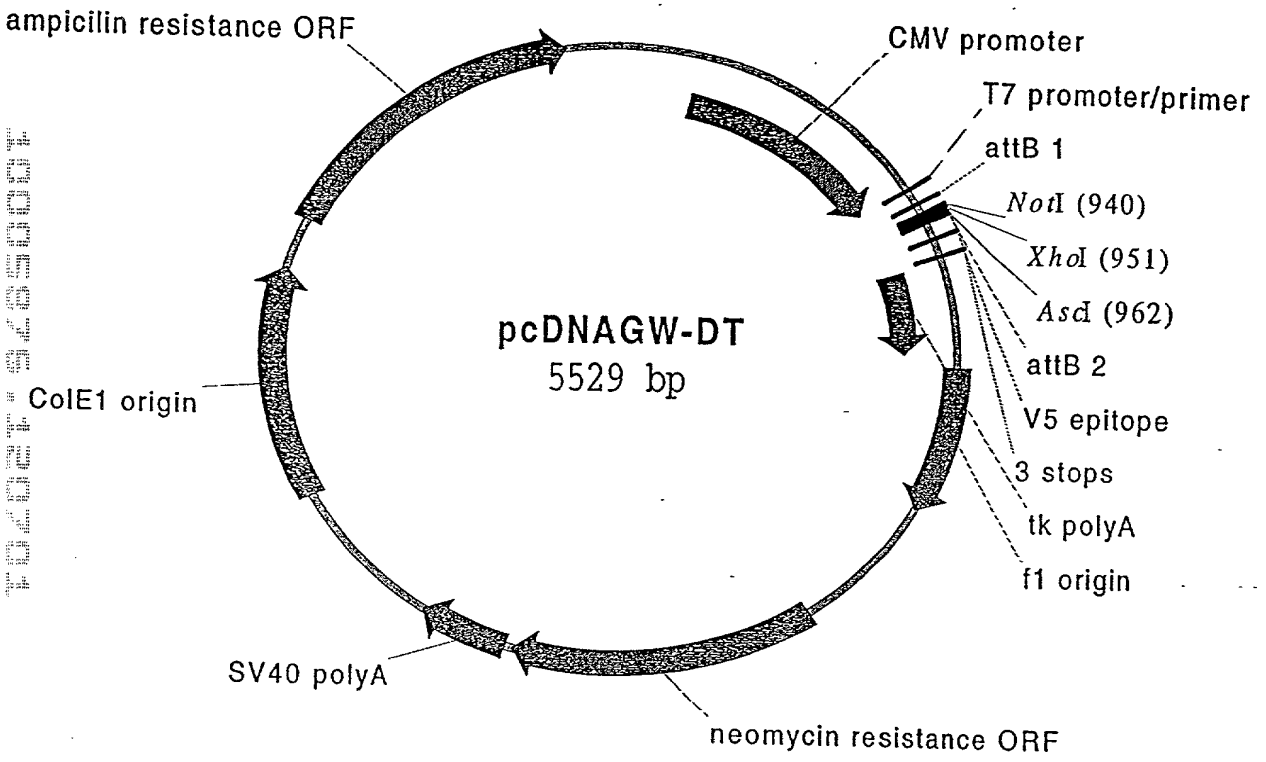

ampicilin resistance ORF
CMV promoter
T7 promoter/primer
attB 1
NotI (940)
XhoI (951)
AscI (962)
attB 2
V5 epitope
3 stops
tk polyA
f1 origin
pcDNAGW-DT
5529 bp
ColE1 origin
SV40 polyA
neomycin resistance ORF

FIGURE 19

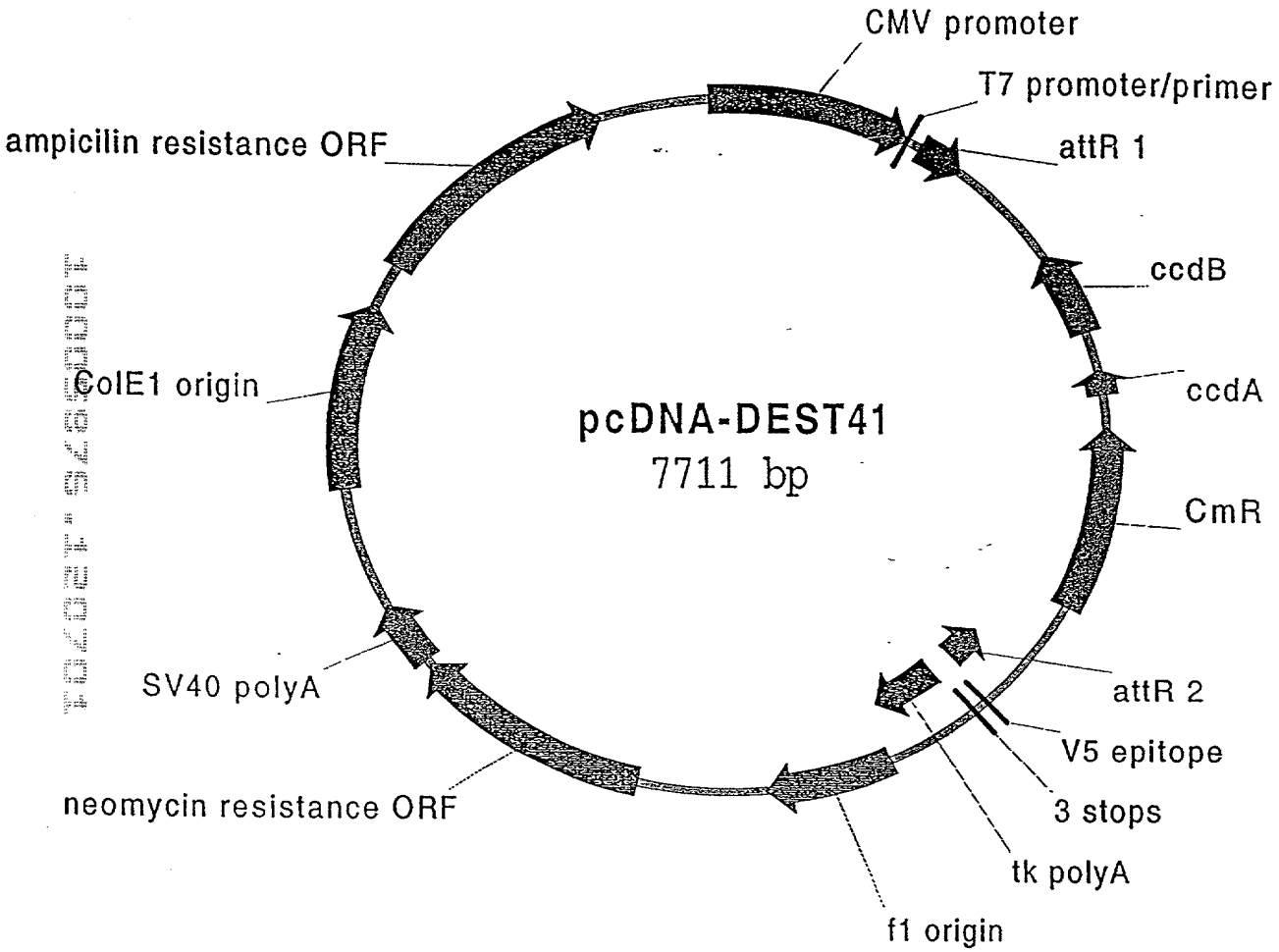

CMV promoter
T7 promoter/primer
attR 1
ccdB
ccdA
CmR
attR 2
V5 epitope
3 stops
tk polyA
f1 origin
neomycin resistance ORF
SV40 polyA
ColE1 origin
ampicilin resistance ORF
pcDNA-DEST41
7711 bp

FIGURE 20

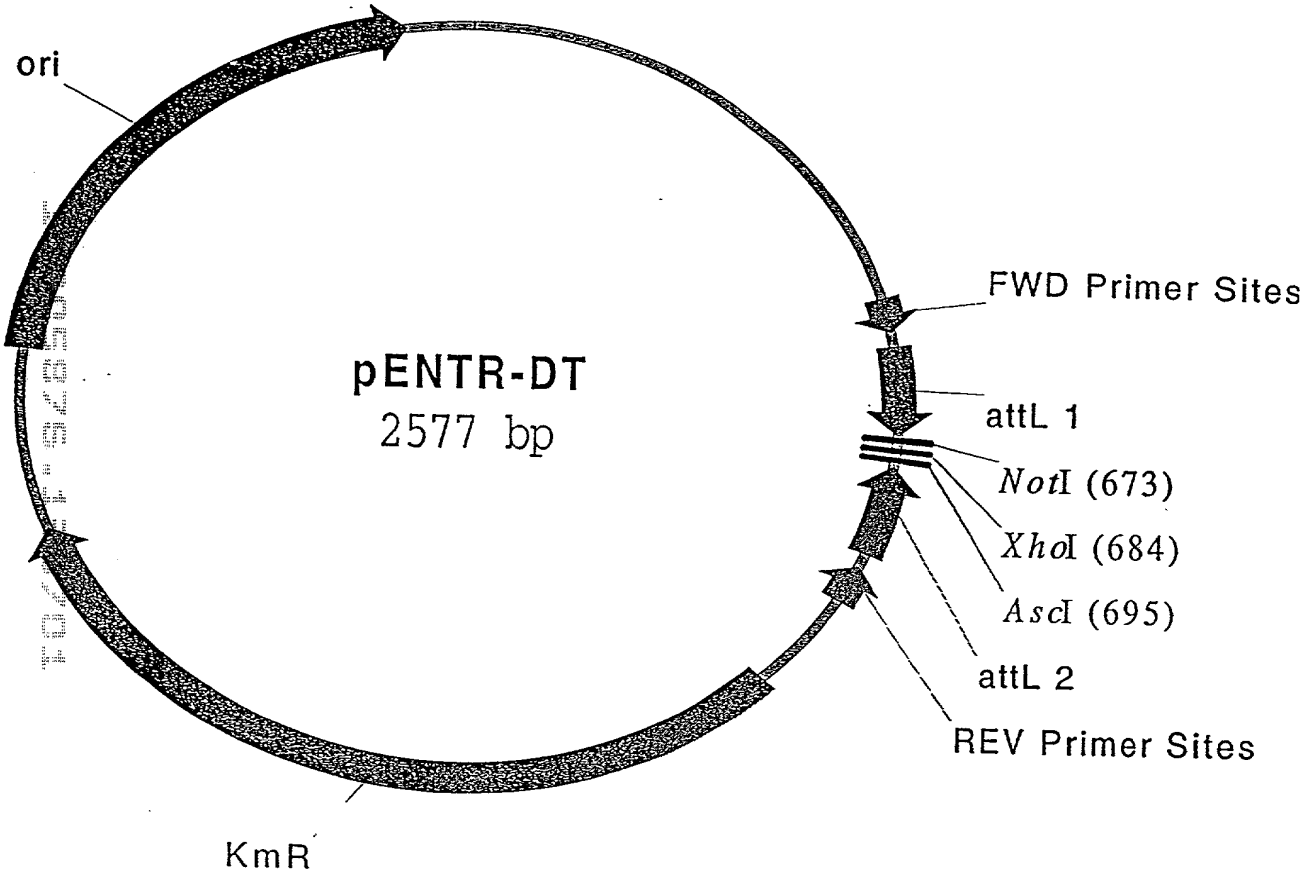

ori
pENTR-DT
2577 bp
FWD Primer Sites
attL 1
NotI (673)
XhoI (684)
AscI (695)
attL 2
REV Primer Sites
KmR

FIGURE 21

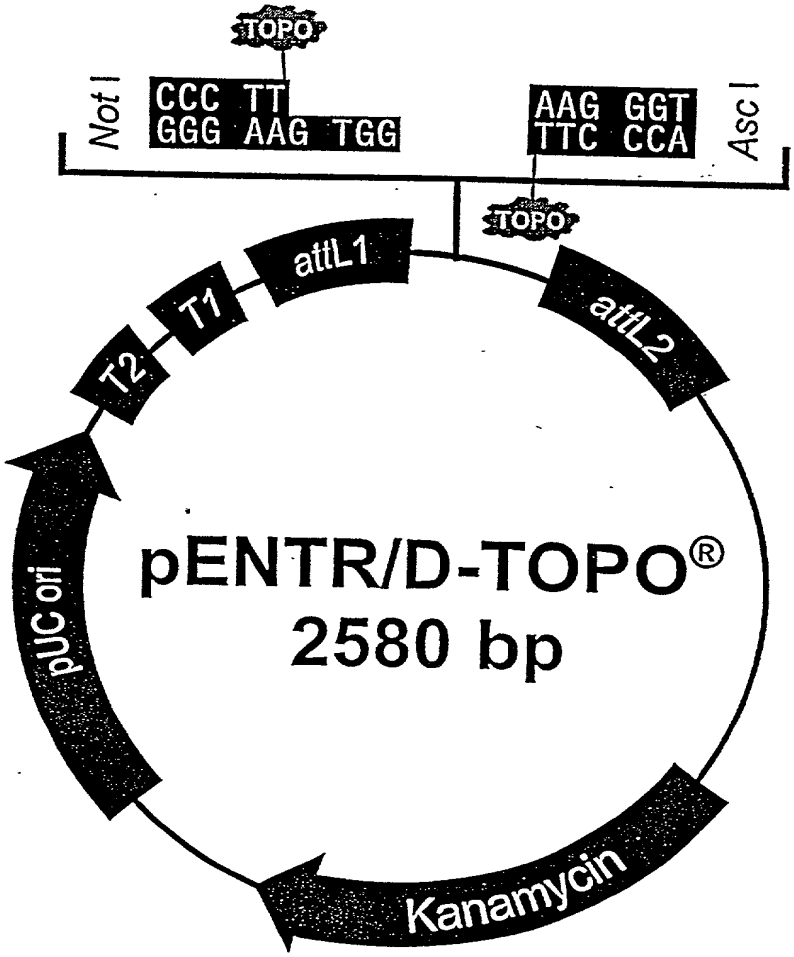

TOPO
Not I
CCC TT
GGG AAG TGG
AAG GGT
TTC CCA
Asc I
TOPO
attL1
T1
T2
attL2
pUC ori
pENTR/D-TOPO®
2580 bp
Kanamycin

FIGURE 22A

```
   1 ctttcctgcg ttatcccctg attctgtgga taaccgtatt accgcctttg agtgagctga
  61 taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg aagcggaaga
 121 gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca
 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
 481 gcagttccct actctcgcgt taacgctagc atggatgttt tcccagtcac gacgttgtaa
 541 aacgacggcc agtcttaagc tcgggcccca aataatgatt ttattttgac tgatagtgac
 601 ctgttcgttg caacaaattg atgagcaatg cttttttata atgccaactt tgtacaaaaa
 661 agcaggctcc gcggccgccc cttcaccatg nnnnnnnnna agggtgggcg cgccgaccca
 721 gctttcttgt acaaagttgg cattataaga aagcattgct tatcaatttg ttgcaacgaa
 781 caggtcacta tcagtcaaaa taaaatcatt atttgccatc cagctgatat cccctatagt
 841 gagtcgtatt acatggtcat agctgtttcc tggcagctct ggcccgtgtc tcaaaatctc
 901 tgatgttaca ttgcacaaga taaaaatata tcatcatgaa caataaaact gtctgcttac
 961 ataaacagta atacaagggg tgttatgagc catattcaac gggaaacgtc gaggccgcga
1021 ttaaattcca acatggatgc tgatttatat gggtataaat gggctcgcga taatgtcggg
1081 caatcaggtg cgacaatcta tcgcttgtat gggaagcccg atgcgccaga gttgtttctg
1141 aaacatggca aaggtagcgt tgccaatgat gttacagatg agatggtcag actaaactgg
1201 ctgacggaat ttatgcctct tccgaccatc aagcatttta tccgtactcc tgatgatgca
1261 tggttactca ccactgcgat ccccggaaaa acagcattcc aggtattaga agaatatcct
1321 gattcaggtg aaaatattgt tgatgcgctg gcagtgttcc tgcgccggtt gcattcgatt
1381 cctgtttgta attgtccttt taacagcgat cgcgtatttc gtctcgctca ggcgcaatca
1441 cgaatgaata acggtttggt tgatgcgagt gattttgatg acgagcgtaa tggctggcct
1501 gttgaacaag tctggaaaga aatgcataaa cttttgccat tctcaccgga ttcagtcgtc
1561 actcatggtg atttctcact tgataacctt atttttgacg aggggaaatt aataggttgt
1621 attgatgttg gacgagtcgg aatcgcagac cgataccagg atcttgccat cctatggaac
1681 tgcctcggtg agttttctcc ttcattacag aaacggcttt ttcaaaaata tggtattgat
1741 aatcctgata tgaataaatt gcagtttcat ttgatgctcg atgagttttt ctaatcagaa
1801 ttggttaatt ggttgtaaca ctggcagagc attacgctga cttgacggga cggcgcaagc
1861 tcatgaccaa aatcccttaa cgtgagttac gcgtcgttcc actgagcgtc agaccccgta
1921 gaaaagatca aaggatcttc ttgagatcct ttttttctgc gcgtaatctg ctgcttgcaa
1981 acaaaaaaac caccgctacc agcggtggtt tgtttgccgg atcaagagct accaactctt
2041 tttccgaagg taactggctt cagcagagcg cagataccaa atactgtcct tctagtgtag
2101 ccgtagttag gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta
2161 atcctgttac cagtggctgc tgccagtggc gataagtcgt gtcttaccgg gttggactca
2221 agacgatagt taccggataa ggcgcagcgg tcgggctgaa cgggggttc gtgcacacag
2281 cccagcttgg agcgaacgac ctacaccgaa ctgagatacc tacagcgtga gcattgagaa
2341 agcgccacgc ttcccgaagg gagaaaggcg gacaggtatc cggtaagcgg cagggtcgga
2401 acaggagagc gcacgaggga gcttccaggg ggaaacgcct ggtatcttta tagtcctgtc
2461 gggtttcgcc acctctgact tgagcgtcga tttttgtgat gctcgtcagg ggggcggagc
2521 ctatggaaaa acgccagcaa cgcggccttt ttacggttcc tggccttttg ctggcctttt
2581 gctcacatgt t
```

FIGURE 22B

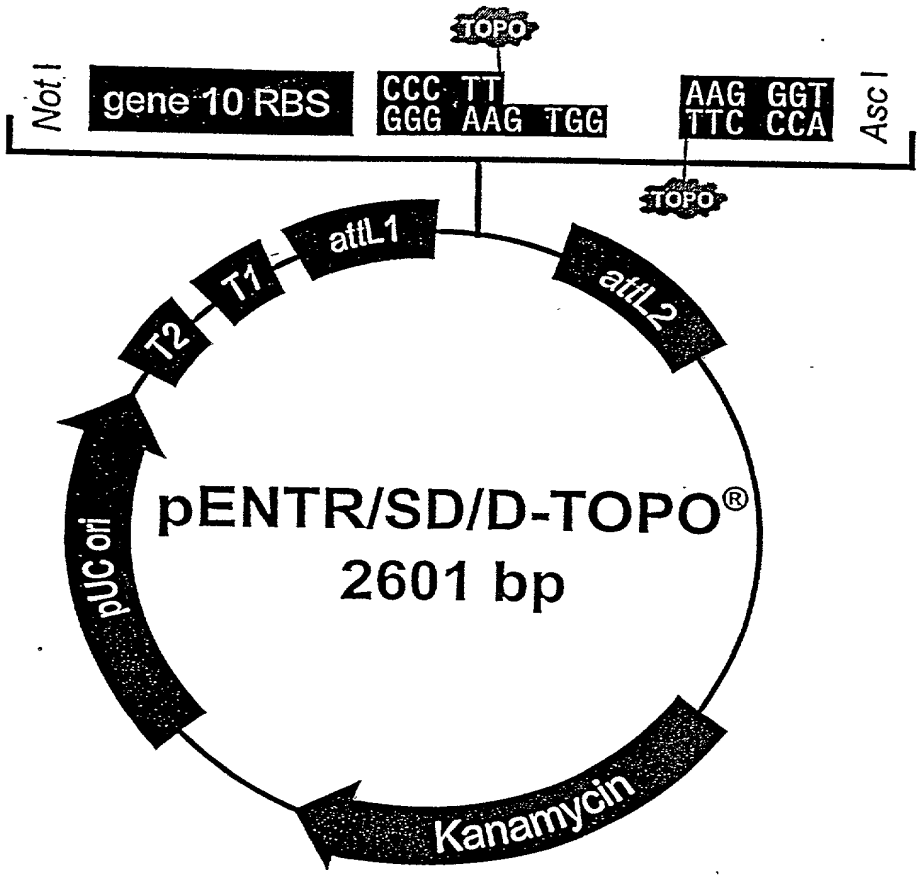

TOPO
Not I
gene 10 RBS
CCC TT
GGG AAG TGG
AAG GGT
TTC CCA
Asc I
TOPO
attL1
T1
T2
attL2
pUC ori
pENTR/SD/D-TOPO®
2601 bp
Kanamycin

FIGURE 23A

```
   1 ctttcctgcg ttatcccctg attctgtgga taaccgtatt accgcctttg agtgagctga
  61 taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg aagcggaaga
 121 gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca
 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
 481 gcagttccct actctcgcgt taacgctagc atggatgttt tcccagtcac gacgttgtaa
 541 aacgacggcc agtcttaagc tcgggcccca aataatgatt ttattttgac tgatagtgac
 601 ctgttcgttg caacaaattg atgagcaatg ctttttttata atgccaactt tgtacaaaaa
 661 agcaggctcc gcggccgcct tgtttaactt taagaaggag cccttcaccn nnnnnaaggg
 721 tgggcgcgcc gacccagctt tcttgtacaa agttggcatt ataagaaagc attgcttatc
 781 aatttgttgc aacgaacagg tcactatcag tcaaaataaa atcattattt gccatccagc
 841 tgatatcccc tatagtgagt cgtattacat ggtcatagct gtttcctggc agctctggcc
 901 cgtgtctcaa aatctctgat gttacattgc acaagataaa aatatatcat catgaacaat
 961 aaaactgtct gcttacataa acagtaatac aaggggtgtt atgagccata ttcaacggga
1021 aacgtcgagg ccgcgattaa attccaacat ggatgctgat ttatatgggt ataaatgggc
1081 tcgcgataat gtcgggcaat caggtgcgac aatctatcgc ttgtatggga agcccgatgc
1141 gccagagttg tttctgaaac atggcaaagg tagcgttgcc aatgatgtta cagatgagat
1201 ggtcagacta aactggctga cggaatttat gcctcttccg accatcaagc attttatccg
1261 tactcctgat gatgcatggt tactcaccac tgcgatcccc ggaaaaacag cattccaggt
1321 attagaagaa tatcctgatt caggtgaaaa tattgttgat gcgctggcag tgttcctgcg
1381 ccggttgcat tcgattcctg tttgtaattg tccttttaac agcgatcgcg tatttcgtct
1441 cgctcaggcg caatcacgaa tgaataacgg tttggttgat gcgagtgatt ttgatgacga
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1681 tgccatccta tggaactgcc tcggtgagtt ttctccttca ttacagaaac ggctttttca
1741 aaaatatggt attgataatc ctgatatgaa taaattgcag tttcatttga tgctcgatga
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1921 agcgtcagac cccgtagaaa agatcaaagg atcttcttga gatccttttt ttctgcgcgt
1981 aatctgctgc ttgcaaacaa aaaaaccacc gctaccagcg gtggtttgtt tgccggatca
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2281 gggttcgtgc acacagccca gcttggagcg aacgacctac accgaactga gatacctaca
2341 gcgtgagcat tgagaaagcg ccacgcttcc cgaagggaga aaggcggaca ggtatccggt
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2461 tctttatagt cctgtcgggt ttcgccacct ctgacttgag cgtcgatttt tgtgatgctc
2521 gtcaggggggg cggagcctat ggaaaaacgc cagcaacgcg gccttttttac ggttcctggc
2581 cttttgctgg ccttttgctc acatgtt
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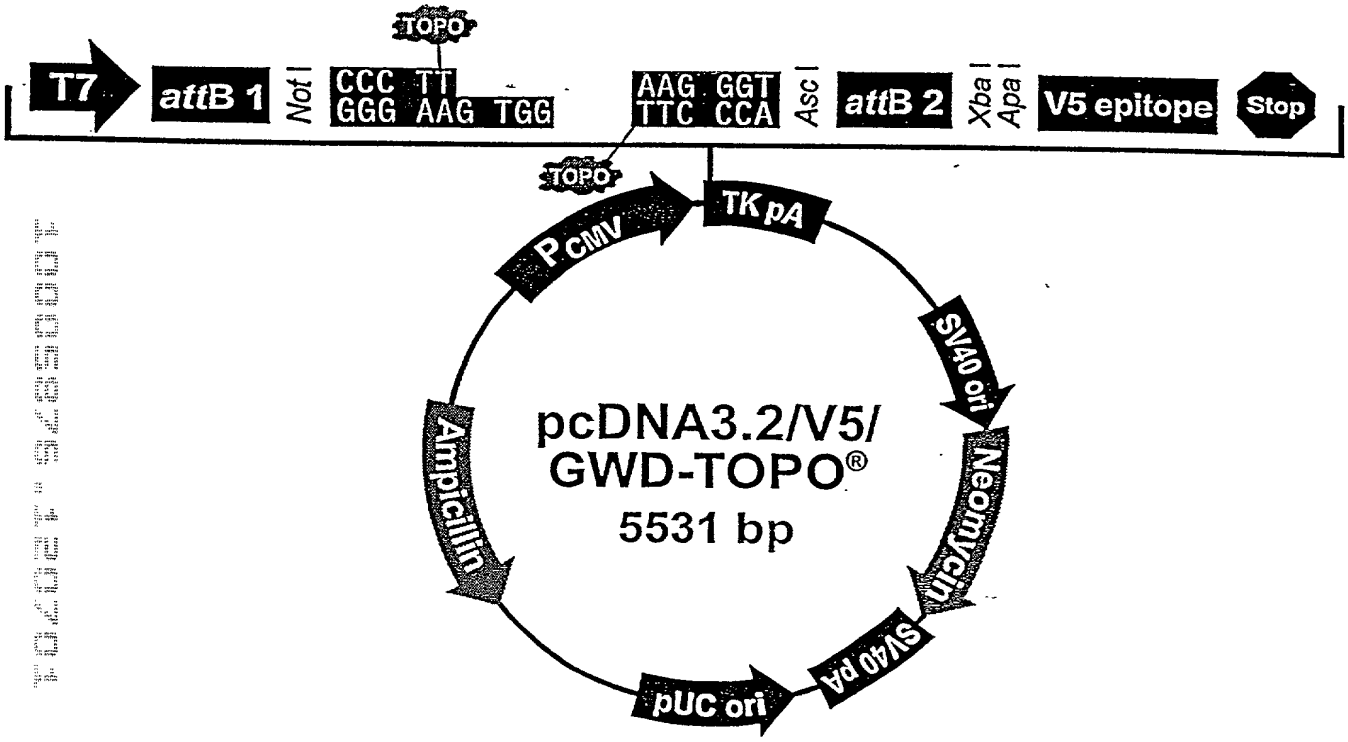
FIGURE 23B


TOPO
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attB 1
Not I
CCC TT
GGG AAG TGG
AAG GGT
TTC CCA
Asc I
attB 2
Xba I
Apa I
V5 epitope
Stop
TOPO
P CMV
TK pA
SV40 ori
Neomycin
SV40 pA
pUC ori
Ampicillin
pcDNA3.2/V5/
GWD-TOPO®
5531 bp

FIGURE 24A

```
   1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
 241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
 481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
 541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
 661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
 721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
 781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
 841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagt
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1081 ggttagtaat gagtttaaac gggggaggct aactgaaaca cggaaggaga caataccgga
1141 aggaacccgc gctatgacgg caataaaaag acagaataaa acgcacgggt gttgggtcgt
1201 ttgttcataa acgcggggtt cggtcccagg gctggcactc tgtcgatacc ccaccgagac
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1381 gcagctgggg ctctaggggg tatccccacg cgccctgtag cggcgcatta agcgcggcgg
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1681 cgttggagtc cacgttcttt aatagtggac tcttgttcca aactggaaca acactcaacc
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2761 caggctcaag gcgcgcatgc ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg
2821 cttgccgaat atcatggtgg aaaatggccg cttttctgga ttcatcgact gtggccggct
2881 gggtgtggcg gaccgctatc aggacatagc gttggctacc cgtgatattg ctgaagagct
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3001 gcgcatcgcc ttctatcgcc ttcttgacga gttcttctga gcgggactct ggggttcgcg
3061 aaatgaccga ccaagcgacg cccaacctgc catcacgaga tttcgattcc accgccgcct
3121 tctatgaaag gttgggcttc ggaatcgttt tccgggacgc cggctggatg atcctccagc-
```

FIGURE 24B

```
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3241 gttacaaata aagcaatagc atcacaaatt tcacaaataa agcatttttt tcactgcatt
3301 ctagttgtgg tttgtccaaa ctcatcaatg tatcttatca tgtctgtata ccgtcgacct
3361 ctagctagag cttggcgtaa tcatggtcat agctgtttcc tgtgtgaaat tgttatccgc
3421 tcacaattcc acacaacata cgagccggaa gcataaagtg taaagcctgg ggtgcctaat
3481 gagtgagcta actcacatta attgcgttgc gctcactgcc cgctttccag tcgggaaacc
3541 tgtcgtgcca gctgcattaa tgaatcggcc aacgcgcggg gagaggcggt ttgcgtattg
3601 ggcgctcttc cgcttcctcg ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag
3661 cggtatcagc tcactcaaag gcggtaatac ggttatccac agaatcaggg gataacgcag
3721 gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa ccgtaaaaag gccgcgttgc
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3901 tcgtgcgctc tcctgttccg accctgccgc ttaccggata cctgtccgcc tttctccctt
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4141 ccactggtaa caggattagc agagcgaggt atgtaggcgg tgctacagag ttcttgaagt
4201 ggtggcctaa ctacggctac actagaagga cagtatttgg tatctgcgct ctgctgaagc
4261 cagttacctt cggaaaaaga gttggtagct cttgatccgg caaacaaacc accgctggta
4321 gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag
4381 atcctttgat cttttctacg gggtctgacg ctcagtggaa cgaaaactca cgttaaggga
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5341 caaaaacagg aaggcaaaat gccgcaaaaa agggaataag ggcgacacgg aaatgttgaa
5401 tactcatact cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga
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5521 cccgaaaagt gccacctgac gtc
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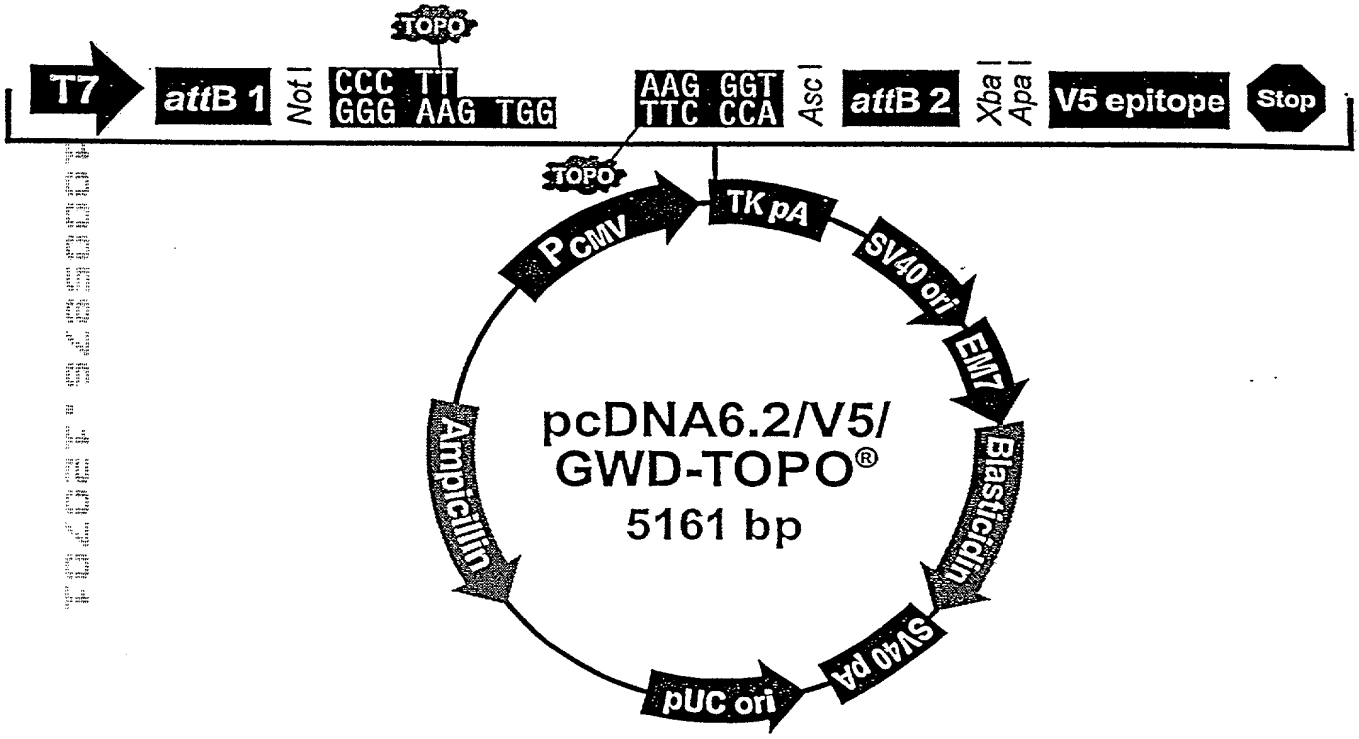
FIGURE 24C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TOPO
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Apa I
V5 epitope
Stop
TOPO
P CMV
TK pA
SV40 ori
EM7
Blasticidin
SV40 pA
pUC ori
Ampicillin
pcDNA6.2/V5/
GWD-TOPO®
5161 bp

FIGURE 25A

```
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  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
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 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt
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 541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
 661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
 721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
 781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
 841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagt
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1021 ccgcggttcg aaggtaagcc tatccctaac cctctcctcg gtctcgattc tacgcgtacc
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1681 cgttggagtc cacgttcttt aatagtggac tcttgttcca aactggaaca acactcaacc
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2941 tggtttgtcc aaactcatca atgtatctta tcatgtctgt ataccgtcga cctctagcta
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3121 ctaactcaca ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa acctgtcgtg
3181 ccagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc-
```

FIGURE 25B

```
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3361 catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt tgctggcgtt
3421 tttccatagg ctccgccccc ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg
3481 gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct ccctcgtgcg
3541 ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc cttcgggaag
3601 cgtggcgctt tctcatagct cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc
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4201 acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc ccgtcgtgta
4261 gataactacg atacgggagg gcttaccatc tggccccagt gctgcaatga taccgcgaga
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5101 atttgaatgt atttagaaaa ataaacaaat aggggttccg cgcacatttc cccgaaaagt
5161 gccacctgac gtc
```
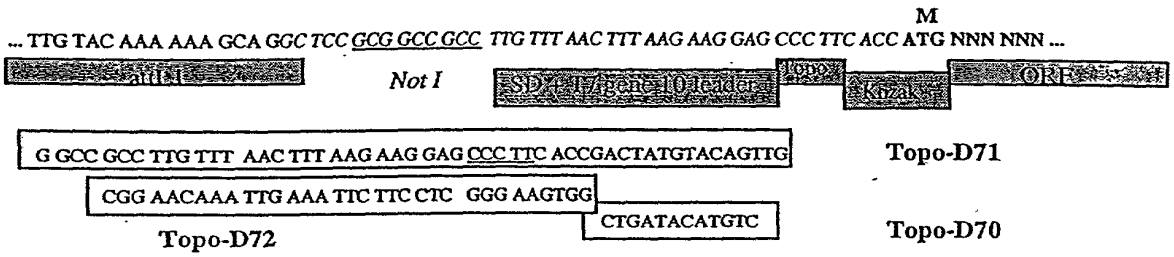
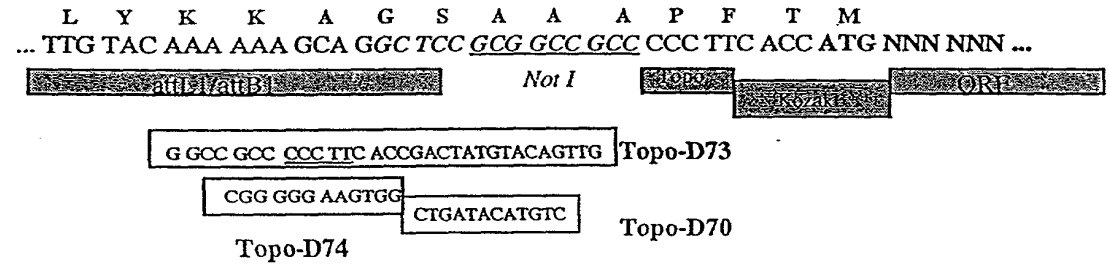

FIGURE 25C

pENTR/SD-dTopo: 5'end



ENTR-dTopo and pcDNAGW-dTopo: 5' end



ENTR/SD-dTopo, pENTR-dTopo, and pcDNAGW-dTopo: 3' end

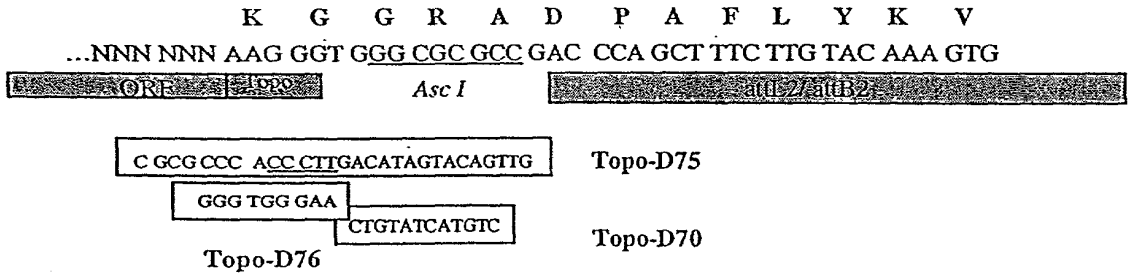


FIGURE 26

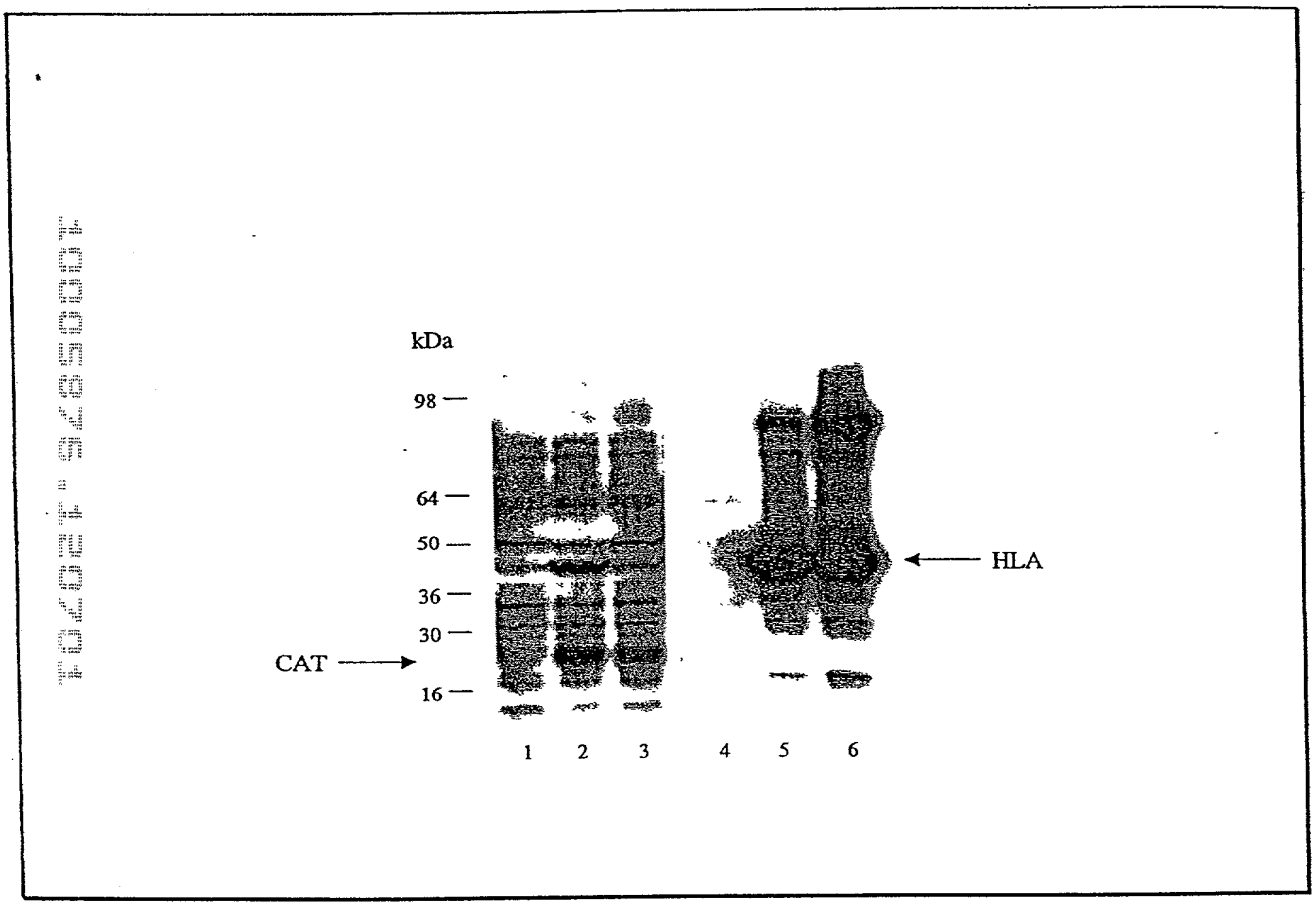


FIGURE 27

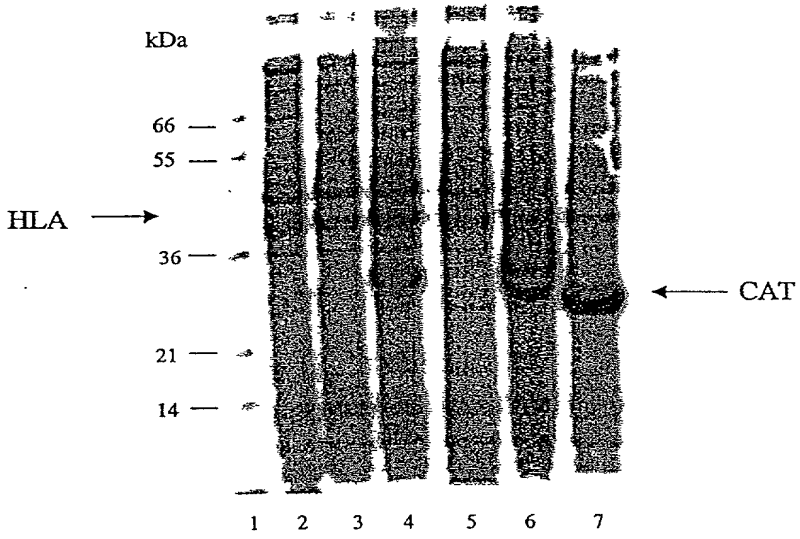


FIGURE 28

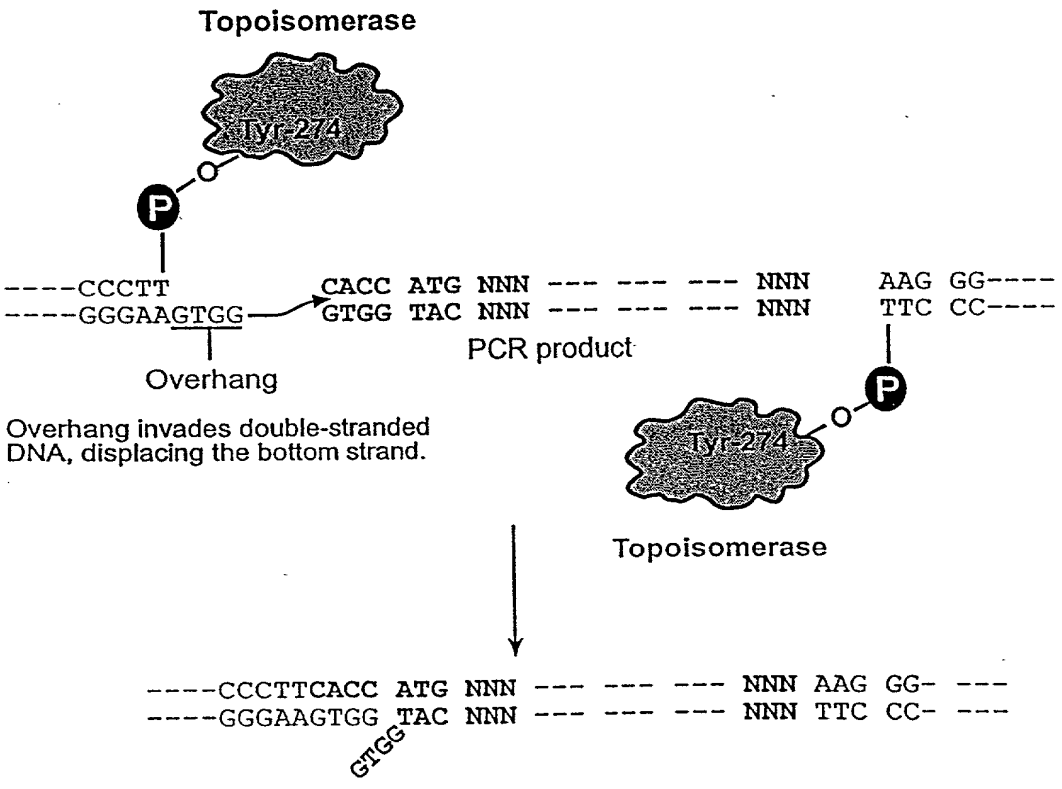
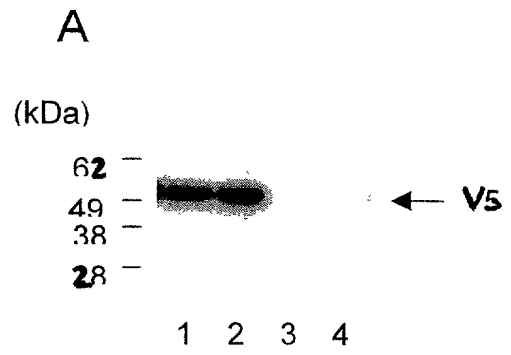
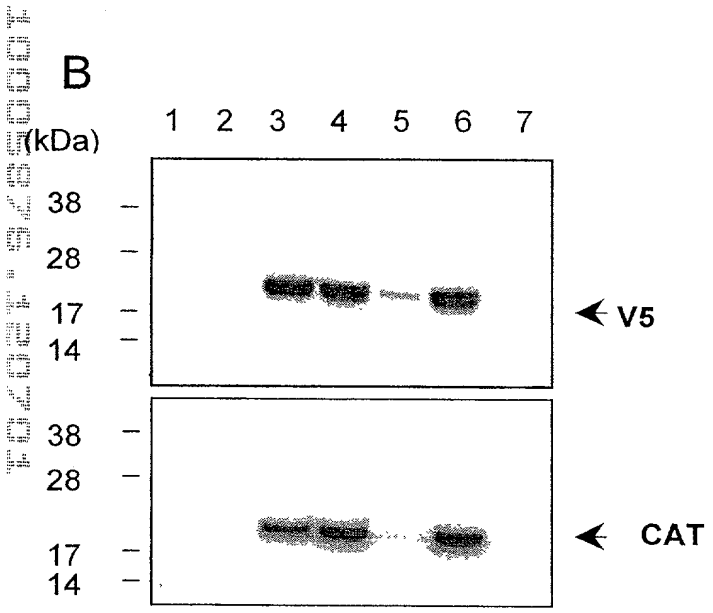
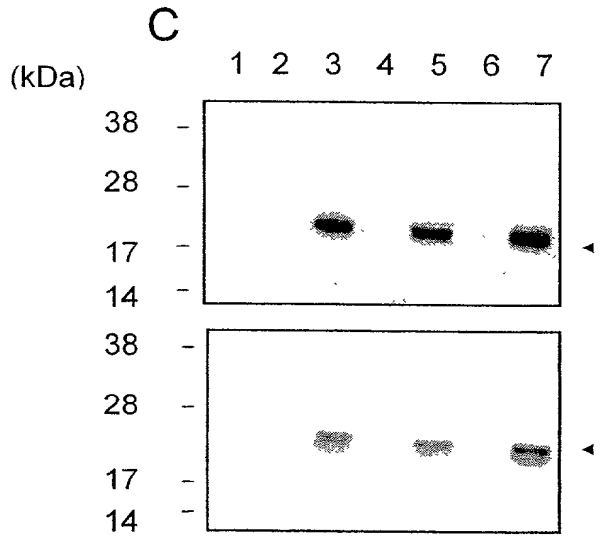

Topoisomerase
Tyr-274
P
O
----CCCTT
----GGGAAGTGG
CACC ATG NNN --- --- --- NNN
GTGG TAC NNN --- --- --- NNN
AAG GG----
TTC CC----
PCR product
Overhang
Overhang invades double-stranded DNA, displacing the bottom strand.
Tyr-274
P
O
Topoisomerase
----CCCTTCACC ATG NNN --- --- --- NNN AAG GG- ---
----GGGAAGTGG TAC NNN --- --- --- NNN TTC CC- ---
GTGG

FIGURE 29



Lane 1: pCMVTetO/CAT/V5TKpA (without secondary PCR)+ Tet
Lane 2: pCMVTetO/CAT/V5TKpA (with secondary PCR)+ Tet
Lane 3: pCMVTetO/CAT/V5TKpA (with secondary PCR) - Tet
Lane 4: pCMVTetO/CAT/V5TKpA (without secondary PCR)– Tet

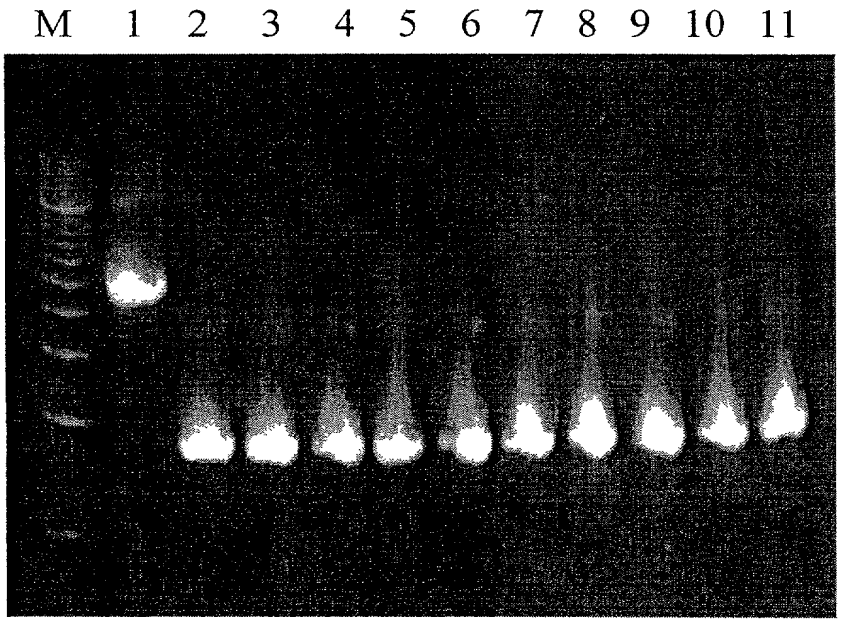




Lane 1: TRex-CHO Cells + Tet
Lane 2: without secondary PCR (with purified CAT) – Tet
Lane 3: without secondary PCR (with purified CAT)+ Tet
Lane 4: without secondary PCR (with unpurified CAT) + Tet
Lane 5: without secondary PCR (with unpurified CAT) – Tet
Lane 6: with secondary PCR + Tet
Lane 7: with secondary PCR -Tet

Lane 1: TRex-293 Cells + Tet
Lane 2: without secondary PCR (with purified CAT) – Tet
Lane 3: without secondary PCR (with purified CAT) + Tet
Lane 4: without secondary PCR (with unpurified CAT) - Tet
Lane 5: without secondary PCR (with unpurified CAT) + Tet
Lane 6: with secondary PCR - Tet
Lane 7: with secondary PCR + Tet

FIG. 30



Lane1: negative control; lanes 2-11: test clones; M: 500 bp marker

FIG. 31

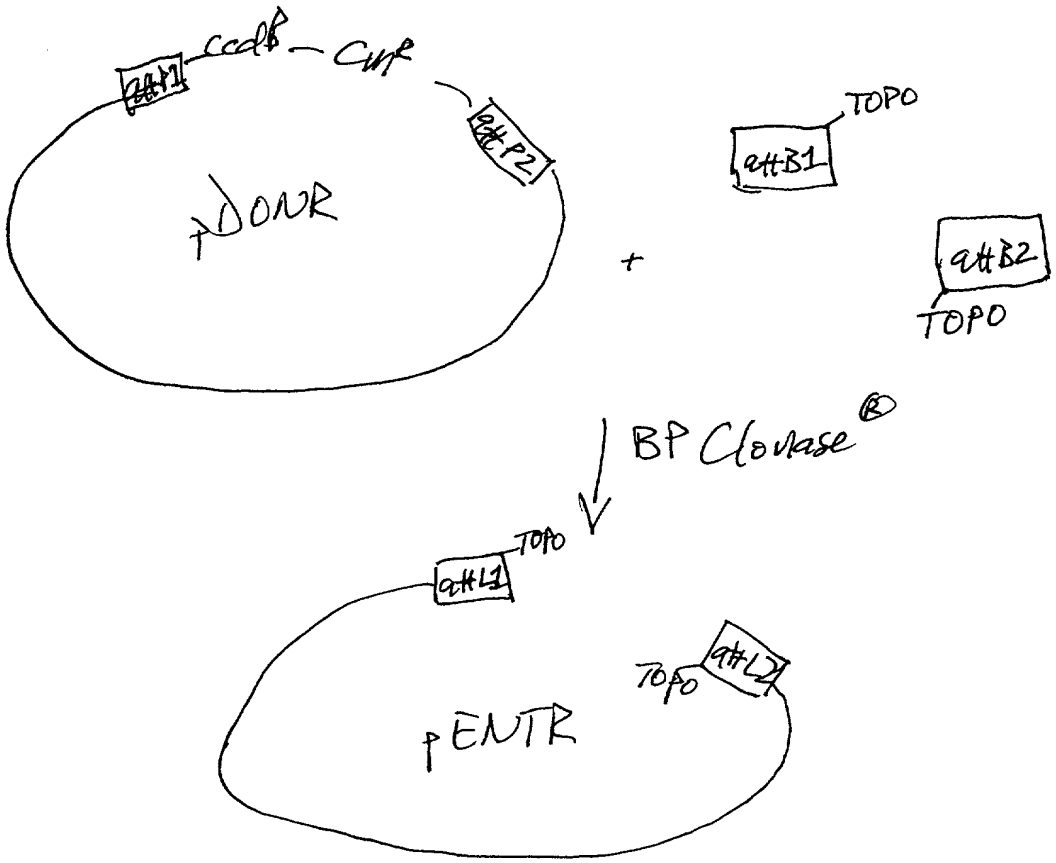

attL1
ccdB – Cm
attL2
pDONR
+
TOPO
attB1
attB2
TOPO
BP Clonase®
TOPO
attL1
TOPO
attL2
pENTR

FIGURE 32

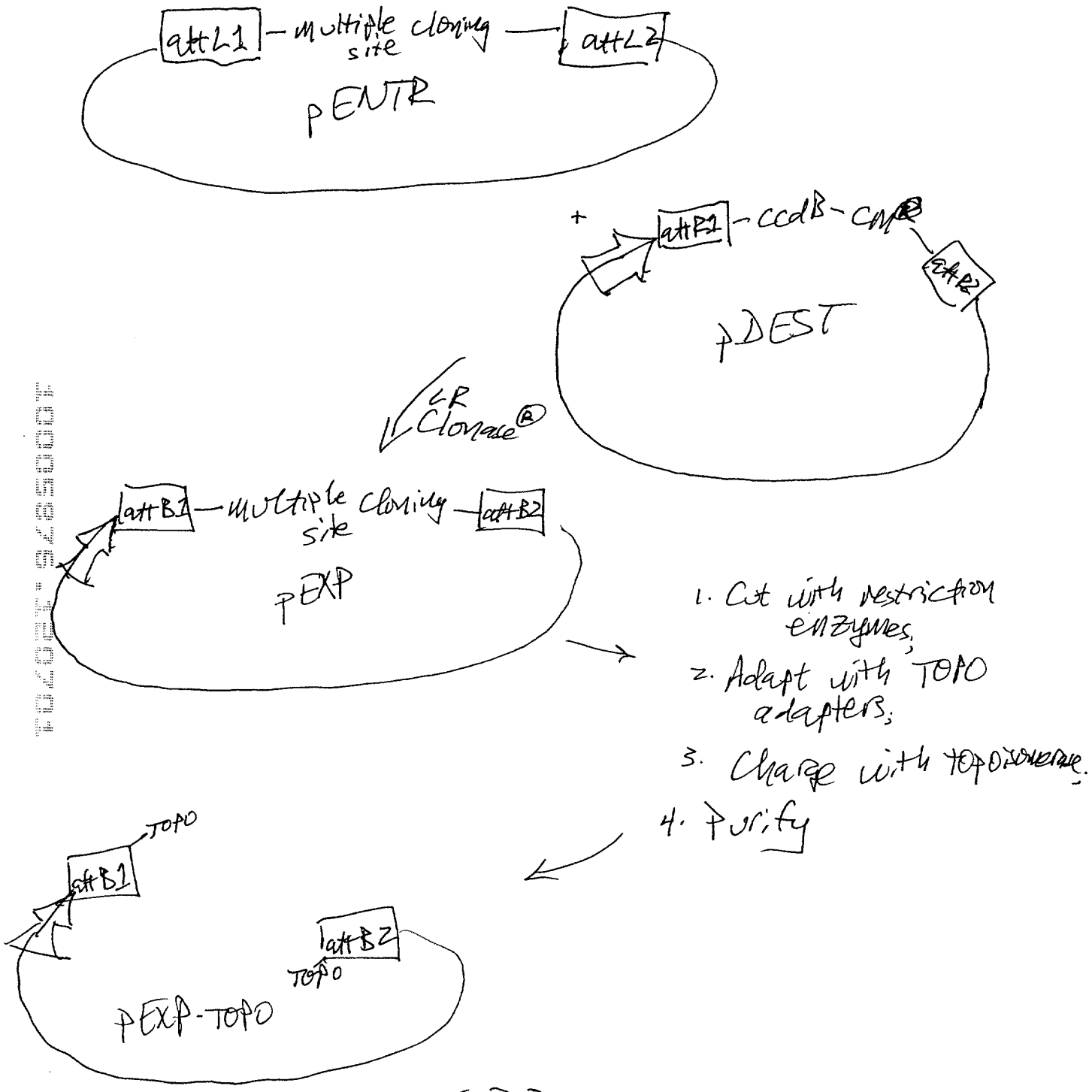

attL1
multiple cloning site
attL2
pENTR
+
attR1
ccdB
CmR
attR2
pDEST
LR Clonase®
attB1
multiple cloning site
attB2
pEXP
1. Cut with restriction enzymes;
2. Adapt with TOPO adapters;
3. Charge with topoisomerase.
4. Purify
TOPO
attB1
attB2
TOPO
pEXP-TOPO

FIGURE 33

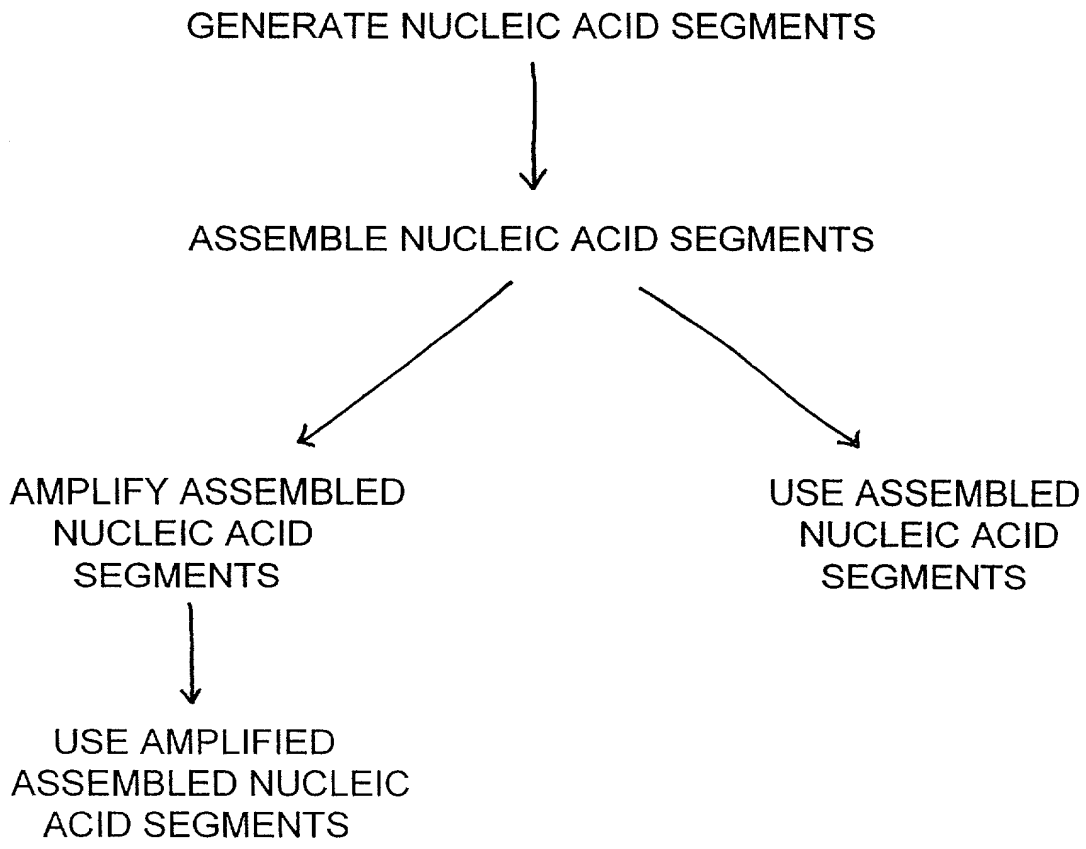

GENERATE NUCLEIC ACID SEGMENTS
ASSEMBLE NUCLEIC ACID SEGMENTS
AMPLIFY ASSEMBLED NUCLEIC ACID SEGMENTS
USE ASSEMBLED NUCLEIC ACID SEGMENTS
USE AMPLIFIED ASSEMBLED NUCLEIC ACID SEGMENTS

FIG. 34

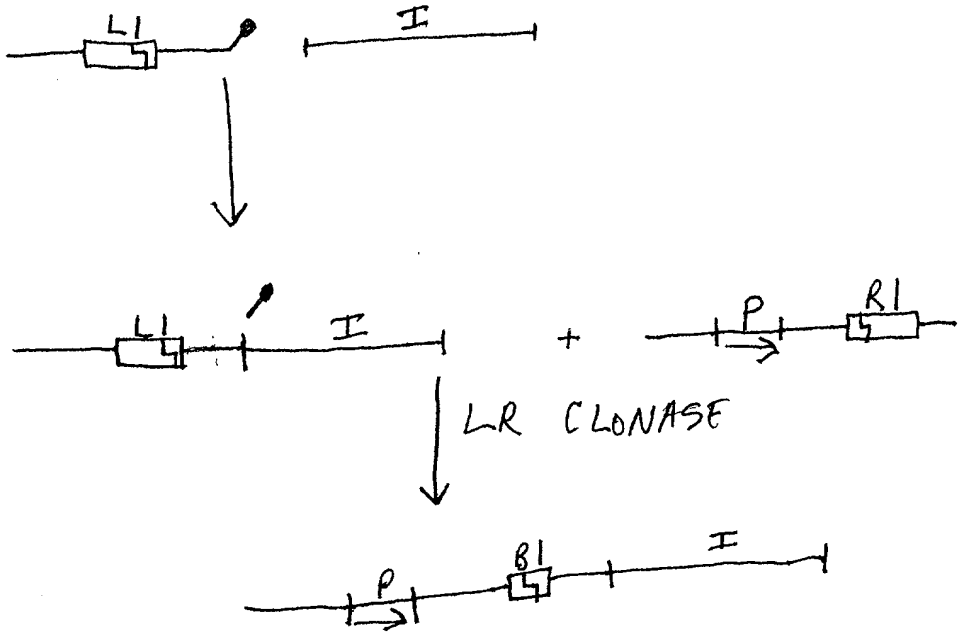


FIGURE 35

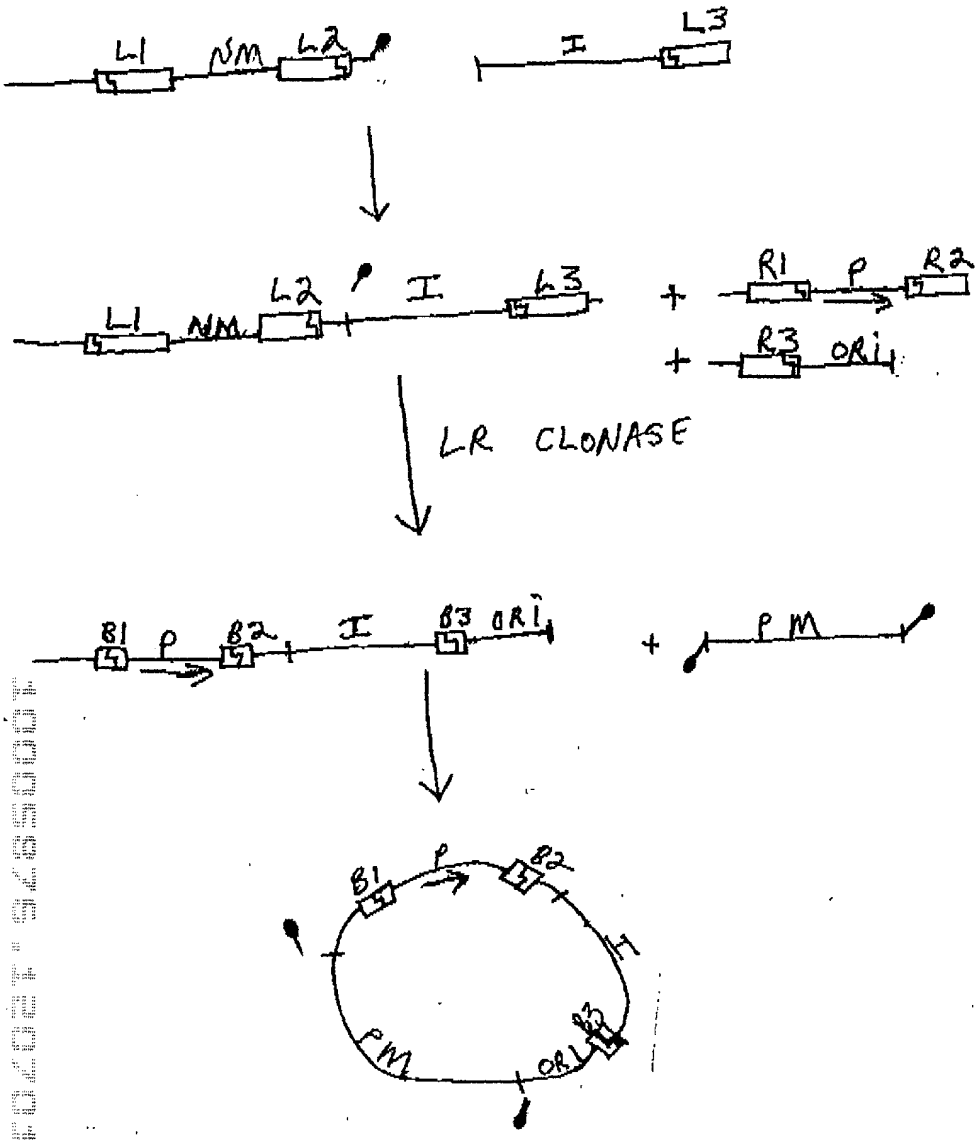

L1
NM
L2
I
L3
L2
I
L3
L1
NM
R1
P
R2
R3
ORI
LR CLONASE
B1
P
B2
I
B3
ORI
PM
B1
P
B2
I
B3
PM
ORI

FIGURE 36

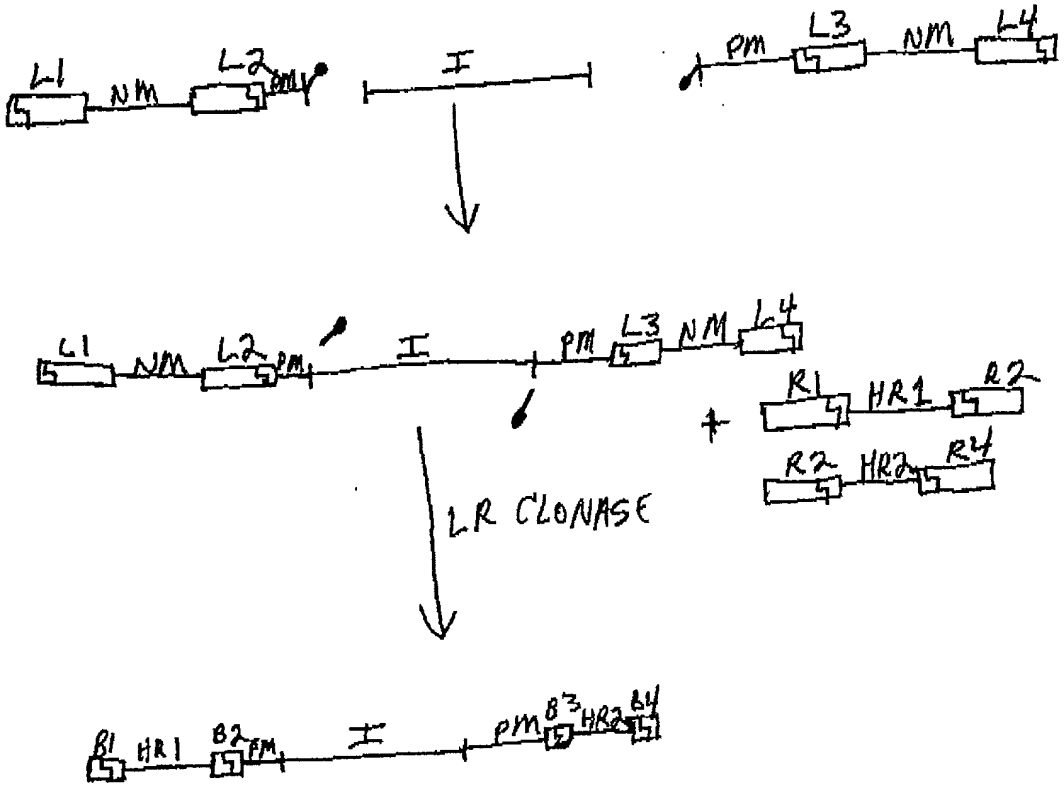


FIGURE 37

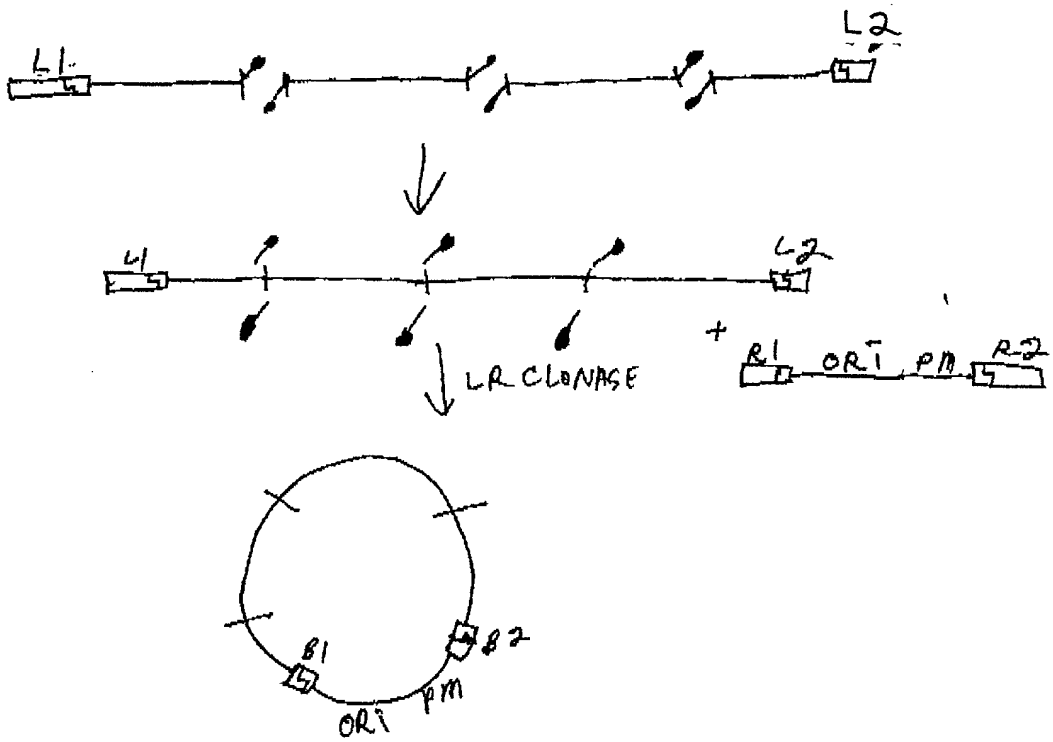


FIGURE 38

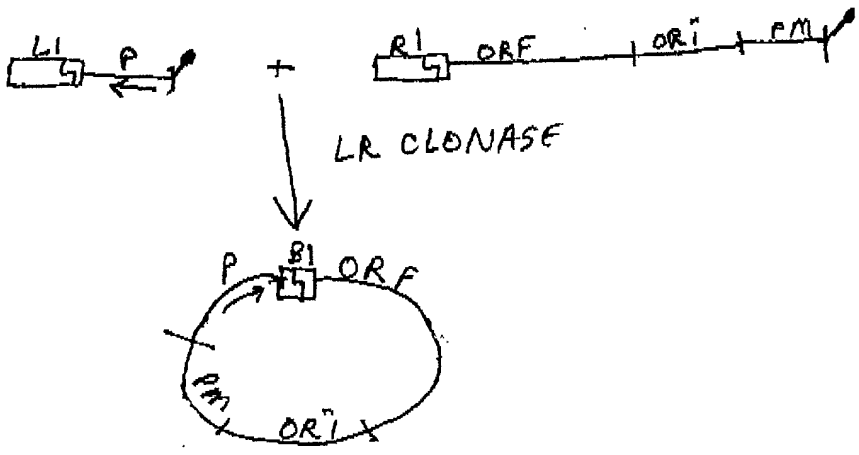


FIGURE 39

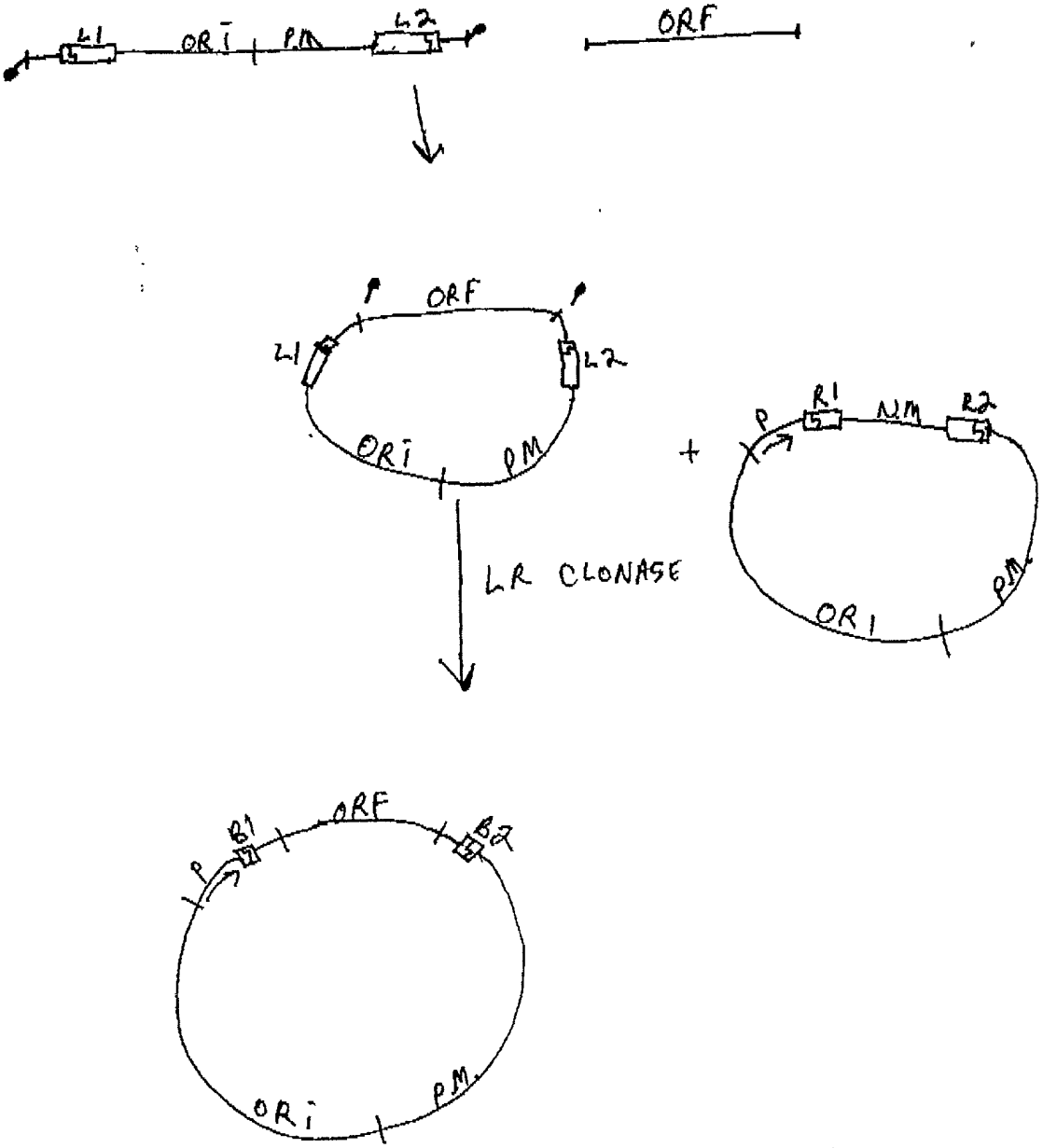

L1
ORI
PM
L2
ORF
ORF
L1
L2
ORI
PM
LR CLONASE
P
R1
NM
R2
ORI
PM
B1
ORF
B2
P
ORI
PM

FIGURE 40